2. West Nile Virus: Molecular Epidemiology and Diversity

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Abstract

West Nile virus (WNV) is a member of the Japanese encephalitis (JE) antigenic complex in the family *Flaviviridae*, genus *Flavivirus*. Since the mid-1990s the incidence of West Nile neuroinvasive disease has increased and the geographic range of the virus has expanded significantly. This collection of widely geographically distributed genotypes has facilitated research on the molecular epidemiology and genetic variation of the virus, and important population parameters such as the mutation rate and natural selective forces. Novel insights have been gained regarding the evolution of a mosquito-borne zoonotic virus on a regional and intercontinental scale. This chapter reviews recent literature on WNV genetic diversity, and discusses research on the phenotypic impact of this diversity on enzootic transmission of the virus and pathogenesis in vertebrates. Also addressed are analyses of viral fitness and intrahost genetic diversity, as well as population genetic determinants that facilitate perpetuation and adaptation of WNV in new environments. Future challenges, including re-analysis of relationships of JE serogroup viruses, and significance of genetic diversity, are posed for consideration.

Keywords

West Nile, Flavivirus, evolution, selection, population dynamics, genetic diversity, molecular epidemiology

1 Overview of WNV Genetic Diversity at a Global Scale

West Nile virus (WNV) is a member of the Japanese encephalitis virush (JEV) antigenic complex in the family *Flaviviridae*, genus *Flavivirus*. Flaviviruses are found on every continent except Antarctica (Gould et al., 2003), but none is as widely distributed as is WNV.

The virus was first detected in the West Nile district of Uganda, Africa, in 1937 (Smithburn et al., 1940) and since then has been isolated from specimens collected in Europe (Hubalek and Halouzka, 1999), Asia (Zeller and Schuffenecker, 2004) and Australia (MacKenzie et al., 1994). Since the mid-1990s the incidence of West Nile neuroinvasive disease has increased and the geographic range of WNV has expanded, with outbreaks occurring in Russia and Europe (Murgue et al., 2002), and the introduction of the virus into the Americas (Lanciotti et al., 1999; Hayes and Gubler, 2005). As the range of the virus has expanded, so has the interest in genetic and antigenic variation as investigators have sought to understand the relationships between geographically disparate WNV-like viruses, and to determine the pattern(s) and impact(s) of viral evolution on a regional and intercontinental scale.

The genetic and antigenic diversity of WNV at the global scale has been addressed for several decades, with rapid recent increases in the level of analytic detail, sophistication and the number of recognized lineages of WNV. Prior to the availability of extensive genetic data on members of the JE serogroup, cross neutralization studies demonstrated that WNV, Kunjin (KUNV) and Koutango (KOUV) viruses are closely related antigenically (Calisher et al., 1989; DeMadrid and Porterfield, 1974). WNV was separated into three largely geographically distinct groups (Africa, India, and Europe and the Middle East) using polyclonal sera and monoclonal antibodies (Blackburn et al., 1987; Hammam et al., 1965). Phylogenetic analyses based on nucleotide sequencing of an approximately 1 kb fragment of the 3′ terminus of the NS5 coding sequence of many of these viruses confirmed the earlier findings, defining genotypes that agreed with the previously proposed antigenic groupings (Kuno et al., 1998). A study undertaken shortly after the introduction of WNV into North America analyzed complete genomes and included several individual WNV strains alongside prototype viruses (Lanciotti et al., 2002). This work proposed two main WNV lineages. Lineage 1 includes three sublineages: (a) distributed in Africa, the Middle East, Europe and the Americas; (b) found in Australia is also known as KUNV; and (c) strains isolated in India. Lineage 2 is generally confined to sub-Saharan Africa and may ocasionally cause outbreaks of encephalitis (Petersen and Roehrig, 2001). There is at least one report of lineages 1 and 2 strains isolated from the same region, i.e., Hungary, in 2003–2004. In this case, lineage 1 virus was associated with encephalitis in geese (Glavits et al., 2005) and mild encephalitis and meningitis in humans (Ferenczi et al., 2005) in Hungary in 2003, followed one year later by isolation of an encephalic lineage 2 virus from a goshawk (*Accipiter gentilis*) in the same region (Bakonyi et al., 2006).

KOUV has not been included in recent phylogenetic studies, but its relationship to the described lineages 1 and 2, was demonstrated antigenically (Varelas-Wesley and Calisher, 1982; Calisher et al., 1989). Charrel and colleagues (Charrel et al., 2003) revisited the phylogeny of Old World WNV strains, including partial coding sequences of KOUV and determined that this agent is a "distant variant" of WNV, in support of the previous antigenic studies. Data presented by these authors did not attempt to incorporate KOUV into the lineage structure proposed by Lanciotti et al. (Lanciotti et al., 2002), but the collected data from genetic and antigenic studies suggest that it is both an independent genetic lineage and an antigenic subtype. Subsequent publications have proposed additional WNV lineages, including "Rabensburg virus," or WNV lineage 3 (Bakonyi et al., 2005), a Russian strain that has been assigned to lineage 4 (Lvov et al., 2004), and a series of Indian strains that comprises lineage 5 (Bondre et al., 2007). KOUV would then be lineage 6.

Although the multiple WNV lineages proposed on the basis of phylogenetic studies are well supported by nucleotide sequence data, considerable confusion remains regarding the proper classification of these lineages as new virus species or WNV subtypes. Various authors have proposed criteria based on nucleotide distances. For example, Kuno and colleagues (Kuno et al., 1998) proposed that > 84% pairwise sequence identity at the nucleotide level be considered the quantitative genetic criteria for inclusion within a species, while Charrel and colleagues proposed a value of > ~79% for inclusion within the species WNV (Charrel et al., 2003). Applying either of these criteria, KOUV (with ~76% identity) and Rabensburg virus (with ~77% identity) would not be classified as WNV, despite demonstrated but limited serologic cross-reactivities (Bakonyi et al., 2005). In light of the increasing number of proposed lineages, and the increasing importance of the JEV serogroup from a public health perspective, an antigenic reexamination of these agents using high quality standardized immune reagents is clearly warranted.

2 Molecular Epidemiology of WNV in the Americas

2.1 Evidence for a Single Point Introduction

The introduction of WNV into North America in 1999 provided a unique opportunity to observe prospectively the outcome of the introduction of an exotic pathogen into a naïve environment. Although the agent was initially misidentified as St. Louis Encephalitis virus (Asnis et al., 2000), it was rapidly recognized on the basis of phylogenetic

analyses and monoclonal antibody binding to be a strain of WNV that was most closely related to a strain that had been isolated in Israel the previous year (Lanciotti et al., 1999). Although the epidemiology and epizootiology of the initial outbreak were consistent with a single recent introduction, it was unclear whether the virus had been introduced in the same year it was initially detected, or whether it had been present at undetectable levels prior to its recognition. The first population study of WNV since its introduction into North America examined envelope (E) coding sequences from eleven WNV strains collected from mosquitoes and birds in New York during the 2000 transmission season (Ebel et al., 2001). This work reported extreme genetic conservation among the strains sampled, with all strains, including a strain collected in 1999, having greater than 99% identity. Some geographic patterns were noted, with a C to U mutation at nucleotide position 1974 occurring in four of five strains collected on Staten Island, suggesting that molecular epidemiologic studies of WNV might be informative in tracking the spread of WNV in North America. From these studies it was concluded that the WNV epidemic/epizootic ongoing in the northeastern US was the result of a single point introduction followed by primary expansion during 1999, overwintering and secondary expansion during 2000 (Ebel et al., 2001). A second population study that included 82 WNV strains confirmed the close genetic relationships of circulating WNV and presented stronger evidence of geographic clustering of strains carrying particular mutations, in this case a C to U mutation at genome position 858. Therefore, early studies of WNV presented data consistent with a single introduction of virus in 1999, and raised the possibility that molecular epidemiologic studies would provide insights into its patterns of perpetuation and dispersal in the Americas.

2.2 Genetic Conservation and Diversification During Colonization

Genetic conservation is typical of most arboviruses (Weaver, 2006; Pisano and Tolou, 1996); however geographic isolation and/or population bottlenecks may lead to diversification, or the virus may adapt to local host populations. The initial studies of WNV collected in the Northeastern US suggested a pattern of conservation, at least during the first two years after introduction (Lanciotti et al., 2002; Ebel et al., 2001). The first study describing WNV sequences collected from a distant point from that of WNV introduction in the Northeastern

United States, included strains collected in Texas (Beasley et al., 2003). Analysis of these strains confirmed the high degree of genetic homogeneity in WNV, reporting a maximum of 0.35% divergence from the NY99 strain. Notably two "sequence subtypes" were detected, one of which differed from NY99 by a U to C substitution at genome position 1442 that resulted in the substitution of an Ala for a Val residue at amino acid position 159 of the envelope protein – a position that had shown some sequence variation among strains collected in Africa and Europe (Lanciotti et al., 2002). It was thus suggested that the amino acid substitution was likely to be a result of genetic drift that occurred during the westward migration of WNV. A further analysis of additional strains collected in Texas established the presence of two clades, one defined by the V to A substitution at E159, and the other defined by five mutations in the PrM-E coding sequences. These clades clustered geographically, with one group of sequences (V to A, E159) clustering in Houston, and the other along a coastal region to the southeast of the main metropolitan area (Davis et al., 2005). WNV isolates in New York also showed evidence of the emergence of strains carrying the mutation resulting in the V to A substitution at E159; this clade was termed "WN02" because it was first noted in New York State during transmission season of the year 2002 (Ebel et al., 2004). The proportion of strains collected in the US that belong to the WN02 genotype increased from 2001 to 2003, suggesting a selective advantage of WN02. WN02 is now the dominant WNV genotype in North America (Davis et al., 2005; Snappin et al., 2007), with the NY99 genotype apparently persisting below the limit of detection of current molecular genetic studies, if at all. Indeed, experimental studies demonstrated that at least two *Culex* species mosquitoes have decreased extrinsic incubation periods for WN02 strains (Ebel et al., 2004; Moudy et al., 2007). Other genetic variants also have been noted. Attenuated strains have been isolated from infected mosquitoes and birds in 2003 in Texas (Davis et al., 2004) and from a phenotypically mixed population isolated from an American crow collected in New York (Jia et al., 2007). Attenuated populations, however, tend to persist in nature for relatively short periods. For example, the attenuated strains detected in Texas in 2003 were not detected in subsequent years (A.D.T. Barrett, personal communication). Thus, during colonization of North America, WNV has remained relatively genetically homogeneous, with the frequent appearance of ephemeral genetic variants, and the rarer emergence of variants (i.e., V159A) that become firmly established.

2.3 Insights into WNV Population Dynamics

Phylogenetic analyses have shown that the most divergent strains of WNV are of African origin (lineage 2), suggesting that the lineage 1 strains probably evolved from lineage 2 ancestors. Since WNV is known to be transported by migratory birds to new locations where competent *Culex* species mosquitoes are available to act as vectors (Malkinson and Banet, 2002), it seems likely that ancestral WNVs dispersed from Africa by this mechanism. WNV in Australia (KUN) and India represent two distinct clades within lineage I, suggesting that after establishment of the virus in a new location, either genetic drift or adaptation to local host populations led to viral genetic change.

The molecular epidemiologic studies that have been conducted since the introduction of WNV into North America, coupled with intensive and extensive WNV surveillance, have facilitated studies examining the population dynamics of WNV as it colonized and adapted to the naïve environment of North America. These studies have shown that the effective population size of WNV (i.e., the effective number of infections, $N_{e\tau}$) increased dramatically in the United States during 2002–2003 (Snappin et al., 2007) (Fig. 1a, b), coincident with a marked spike in the number of human cases (Hayes and Gubler, 2005) (Table 1). This increase in activity is widely attributed

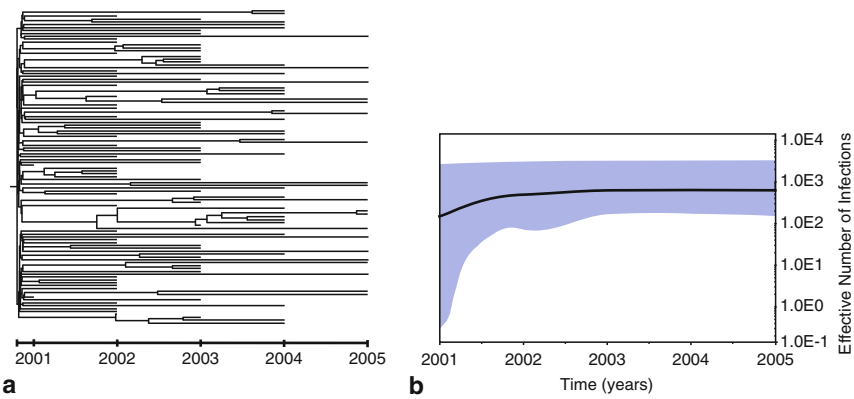

Figure 1. **a** Maximum a posteriori phylogenetic tree of 110 WN02 genotype viruses from samples obtained during the period from 2001 to 2005. For all branches, the times assigned to each tip correspond to the dates of sampling. **b** Bayesian skyline plot for the WN02 genotype. The *bold line* represents the median estimate of the effective number of infections through time, with the 95% HPD values shown in the *shaded area*. The effective number of infections, a measure of relative genetic diversity, is given as $N_e\tau$, where N_e is the effective population size and τ is the generation time. (Snappin et al., 2007, with permission by the American Society for Microbiology.)

Table 1. Human West Nile disease cases 1999–2007[a] United States.

Year	ND[b]	Fever	Unspecified symptoms	Total cases	Deaths
1999	59	3	0	62	7
2000	19	2	0	21	2
2001	64	2	0	66	9
2002	2,946	1,160	50	4,156	284
2003	2,860	6,830	166	9,856	264
2004	1,142	1,269	128	2,539	100
2005	1,294	1,607	99	3,000	119
2006	1,459	2,616	194	4,269	177
2007	1,227	2,350	53	3,630	117
Total	10,973	15,704	696	27,373	1,060

[a]Reported to CDC, as of December 21, 2007 (http://www.cdc.gov/ncidod/dvbid/westnile/)
[b]Neuroinvasive disease including meningitis, encephalitis, acute flaccid paralysis

to the westward expansion of the WNV epizootic to the Rocky Mountains and the midcenter of the US, but increases in activity were also noted during this period in regions such as New York, where WNV activity was already well established (Lukacik et al., 2006). Peak prevalence of WNV in North America was reached in 2003, after which both the WNV population and the number of human cases remained fairly constant. Notably, the rapid population growth of WNV during 2002–2003 was coincident with the V159A mutation becoming fixed in North American WNV populations. The evolutionary rate of WNV has been estimated, and is approximately 5×10^{-4} substitutions per site per year (Snappin et al., 2007; Bertolotti et al., 2007). Several investigators have demonstrated increases in pairwise viral nucleotide diversity over time (Bertolotti et al., 2007; Ebel et al., 2004) suggesting an expanding pool of available WNV genotypes in nature (Fig. 2). Coupled with an increasing rate of anthropogenic environmental change, this pool of WNV genotypes seems likely to insure continued virus perpetuation in North America.

2.4 Sampling Bias and Methodological Issues: Impact on Conclusions

Observational studies are frequently plagued by questions regarding the sampling strategy and criteria for strain inclusion. Specifically, it is difficult to determine whether molecular epidemiologic studies are biased because the investigator has focused sampling on a particular type of host, a specific location, etc. For example,

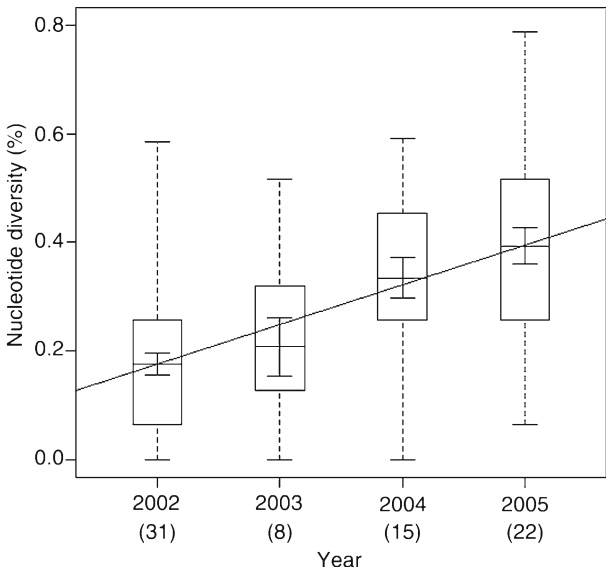

Figure 2. Genetic diversity in WNV from the Midwestern USA, 2002–2005. Genetic diversity is expressed as nucleotide diversity (Nei, 1987), or mean proportion of nucleotide differences among sequences within each year, corrected for multiple substitutions using a model of molecular evolution derived from a hierarchical likelihood ratio test approach implemented using the computer program Modeltest, version 3.7 (Posada and Crandall, 2001). *Lines* and *small error bars* indicate means and standard errors, respectively. Boxes indicate third quartiles, and large error bars indicate ranges. The *solid line* is a least squares regression line indicating the rate of increase of viral genetic diversity over time ($R2 = 0.3626$, $t = 21.69$, $P < 0.0001$). Sample sizes for each year are indicated in parentheses (Bertolotti et al., 2007, with permission from Elsevier Limited).

dead birds serve as excellent sentinels of WNV activity in North America. However, sequences drawn from dead bird samples may represent only the most pathogenic strains in a focus. One early study (Ebel et al., 2001) attempted to address this issue by including samples form both mosquitoes and birds. No signature mutations were observed in this or other studies (Anderson et al., 2001; Beasley et al., 2003) suggesting that both mosquito- and avian-derived sequences represent an essentially random sample of the WNV strains present in a sampling site, thereby increasing confidence that sampling strategy has not significantly biased sequence-based studies of WNV population biology. However, no study has yet included a sufficient number of complete genome sequences from divergent hosts and applied a sufficiently powerful analytic method (i.e., to detect subtle "signatures") to rule out the possibility that bias has

been introduced. Importantly, several groups of investigators, using a wide array of WNV sequence sets derived from mosquitoes, birds and humans (Herring et al., 2007) have come to similar conclusions regarding the basic population dynamics of WNV.

2.5 Implications for WNV Pathogenesis

Viral genetics impact pathogenesis. The genetic variation that exists in naturally occurring WNV populations is thus clearly of central importance in determining the disease burden that the virus places on birds, horses and human beings. Several virally-encoded determinants of pathogenesis are variable in natural populations. One well characterized determinant is the glycosylation state of the envelope (E) protein. Most US strains isolated to date are glycosylated at position 154 in the E protein, as are many of the WNV strains responsible for more severe outbreaks of WN disease. Interestingly, this glycosylation site is relatively well conserved among other flaviviruses (Ryman et al., 1997). Studies using reverse genetics have shown that glycosylation of the E protein leads to a more neuroinvasive phenotype in mice than non-glycosylated strains, although viruses lacking E protein glycosylation retain neurovirulence (Beasley et al., 2005; Shirato et al., 2004). E protein glycosylation also appears to play a role in attachment and entry of WNV into host cells, with non-glycosylated viruses attaching to mosquito cells at rates up to 30-fold more efficiently than glycosylated viruses *in vitro*. Overall replication rates of glycosylated and non-glycosylated viruses, however, were similar (Hanna et al., 2005). In seeming contrast to these results, deletion of the glycosylation site in the WNV E protein and the homologous site in tick-borne encephalitis virus resulted in substantially decreased viral particle release from mammalian cells (Goto et al., 2005; Hanna et al., 2005). WNV isolated from *Anopheles maculipennis* mosquitoes in 1971 in Portugal, associated with an equine epizootic, was non-glycosylated (Parreira et al., 2007). Serosurvey indicated a low level of transmission of this non-glycosylated variant, suggesting that E protein glycosylation may be associated with widespread transmission of WNV.

Additional determinants of pathogenesis are also variable in nature. Mutations in the NS2a and prM genes in members of the viral swarm of an American crow isolate led to attenuation in infection of mosquitoes and birds, but the variants reverted to a wild type phenotype after infection of these hosts (Jia et al., 2007). Recently, a

single amino acid residue at position 249 of the NS3 helicase was implicated in WNV pathogenesis in American Crows (Brault et al., 2004, 2007), but not house sparrows (Langevin et al., 2005). Nonetheless, this substitution of a proline for other amino acids at position 249 of the NS3 has been observed in many outbreaks throughout the world and appears to be subject to positive selection (Brault et al., 2007). Further, mouse attenuated, small plaque, temperature sensitive WNV variants were detected in Texas in 2003 (Davis et al., 2004). Studies of mouse neuroinvasiveness and neurovirulence indicated that several of these isolates were attenuated in neuroinvasiveness, but all were neurovirulent. Variable nucleotide changes were detected, suggesting that more than one set of nucleotide changes may lead to attenuation. Interestingly, while several WNV strains have been isolated that are decreased in their ability to produce disease in selected *in vivo* laboratory models, none has yet been detected that is significantly more pathogenic than the NY99 strain of WNV. This suggests that this strain represents a maximum in the pathogenic potential for this virus, from which reductions are significantly easier to achieve compared with increases.

3 Ecology and Phylogeny: WNV Adaptation in the Western Hemisphere

3.1 General Considerations

Arboviruses perpetuate in nature by replication in alternating hosts, i.e., blood-feeding arthropods and vertebrates. Their population dynamics are therefore dependent on those of at least two other species. Mosquito populations may be unstable due to temperature and rainfall patterns that vary both within and between seasons. Similarly, bird populations vary in abundance due to several factors, including seasonal migration and roosting behaviors. Generally, however, perpetuation of mosquito-transmitted infections depends on the vectorial capacity (VC) of host mosquito populations. Vectorial capacity is essentially an entomological restatement of the "basic reproductive rate" (R_0) of a pathogen, referring to the number of secondary infections expected to occur from the introduction of a single infection in a naïve population. An equation formalizing VC was described by MacDonald (1961) and later modified by others. One of these, described by Black and Moore (1996), provides a useful platform for rational examination of selective forces that may shape WNV (and other arboviruses). This formula is

$$VC = \frac{ma^2 p^n b}{-In(p)}$$

where VC is vectorial capacity (R_0); m is vector density in relation to the host; a is the probability that a vector feeds on a host in 1 day (i.e., the host preference index × feeding frequency); p is the probability that a vector survives one day; n is the duration of the extrinsic incubation period (EIP) in days; b is vector competence (the proportion of vectors ingesting an infective meal that are later able to transmit the infection; and $1/(-\ln(p)$ is the duration of the vector's life in days after surviving the EIP. This equation demonstrates that the abundance (m) and vector competence (b) of mosquito populations would impact the reproductive rate of WNV linearly and thus relatively weakly. In contrast, host feeding (a), vector longevity (p) and EIP (n) would impact R_0 much more powerfully (e.g., as a square or exponent.) It seems to follow that virus infectivity for mosquitoes, which would be incorporated into VC as b, would be of relatively minor importance relative to viral factors such as the speed of dissemination from the midgut that would impact the duration of the EIP, which would influence VC as n. Thus, natural selection might favor a poorly infectious but rapidly disseminating virus over a highly infectious virus that disseminates slowly. Similar predictions might be made about viral influences on other mosquito-associated factors such as host preference, survivorship, etc.

Arboviruses that exist in temperate environments also must adapt to the seasonal activity of their hosts. WNV, which is generally believed to have originated in Sub-Saharan Africa, readily survives the harsh winters in the northeastern and north central US. This seems to be facilitated by vertically infected, diapausing adult female mosquitoes (Nasci et al., 2001). Experimental studies with flaviviruses demonstrate that the rates of successful vertical transmission are extremely low [reviewed in (Rosen, 1987)]. Although some studies of WNV using intrathoracically inoculated *Culex tarsalis* indicate minimum filial infection rates as high as 6.9 (Goddard et al., 2003), the relevance of these findings to the natural transmission cycle, where mosquitoes are infected perorally, is unclear. While it is conceivable that overwintering following vertical transmission could create a population bottleneck wherein genetic drift might become important, the success of WNV over several years in temperate North America suggests that bottlenecks may in fact be rare and demonstrates the phenotypic robustness of this virus.

3.2 Adaptation to Mosquito Hosts

The general considerations discussed above suggest that, in theory, WNV would be expected to rapidly maximize the efficiency of the mosquito phase of its life cycle. Strong evidence now has been presented that this has occurred. Phenotypic studies were undertaken comparing WNV strains belonging to the NY99 and WN02 genotypes. These studies demonstrated that strains belonging to the WN02 genotype were transmitted more efficiently, and transmission was evident two days earlier than with those belonging to the NY99 genotype (Ebel et al., 2004; Moudy et al., 2007). This reduction in EIP results in increased vectorial capacity of WN02-infected mosquitoes relative to those infected with NY99. In subsequent studies evaluating a range of extrinsic incubation temperatures, the difference in transmission between the two viral genotypes increased with temperature and days since feeding, with a 2% advantage at day 4 and a 3% advantage at day 6 at 20°C, but a 10% advantage at day 4 and a 14% advantage at day 6 at 30°C (Kilpatrick, 2008). This advantage in transmissibility has led to WN02 apparently displacing NY99-like viruses; WN02 is currently dominant and apparently stable throughout North America (Davis et al., 2005). Therefore, the pattern of relative evolutionary stasis that characterized the early years of the WNV outbreak in North America was punctuated by a period of rapid evolution, where a newly emergent genotype (WN02) rapidly spread throughout North America, displaced other WNV clades and achieved peak prevalence (Snappin et al., 2007).

3.3 Adaptation to Avian Hosts

Significant avian mortality has been a notable, but not entirely unique, feature of WNV in North America as avian mortality has been noted elsewhere (Kramer and Bernard, 2001; Ladeau et al., 2007; Bin et al., 2001). It might have been predicted that the virus and host populations would co-evolve toward a less pathogenic interaction in a manner similar to myxomavirus following introduction to Australia (Anderson and May, 1982). In the case of WNV, however, this expectation has several conceptual flaws. First, significant avian mortality is not as universal as is typically stated. In the neotropics, for example, little mortality has been noted, possibly due to cross protection from other *Culex* transmitted flaviviruses circulating in the region such as St. Louis encephalitis, Ilheus, or Rocio viruses. Further, experimental infections of numerous avian species have shown significant intra- and inter-specific

variability in response to infection and even more variation between avian families (Komar et al., 2003). Therefore, it seems unlikely that avian mortality acts strongly to shape WNV populations because it is at best an inconsistent phenomenon. Moreover, there is not yet clear evidence of viral adaptation to birds in North America, or that birds have yet developed resistance to WNV. Intriguingly, Brault et al. demonstrated that a single amino acid substitution in the WNV NS3 protein that resulted in increased viremia in the American crow (Brault et al., 2007) was positively selected. This finding suggests that Corvids are of unique and paramount importance in the worldwide emergence of WNV, and would seem to challenge most existing models of the transmission dynamics of arthropod-borne viruses. Comparative infection studies with Old World and New World passeriform birds and WNV strains and with native and tropical passeriform birds with WNV isolated in the US and in tropical Americas will be helpful in clarifying whether adaptation to birds occurs in nature, and more clearly define the role of these hosts in shaping WNV population structure.

4 Evolutionary Mechanisms in West Nile Virus

Molecular epidemiologic and experimental studies on genetically defined WNV strains, described above, have clearly established that the virus has undergone evolutionary change in the eight years since it was introduced into North America. It therefore is valuable to examine in detail the evolutionary mechanisms that have led to this change in order to understand the extraordinary success of WNV in adapting to a new environment. Accordingly, recent studies have investigated mechanisms that may be important in shaping WNV populations. These include various types of natural selection, intrahost population dynamics, host switching and viral fitness. Importantly, and as revealed through some of the studies published to date, it is extremely difficult to isolate these processes from one another because they appear to be related. For example, natural selection and infection of a particular host might simultaneously affect intrahost population dynamics and viral fitness, leading to difficulty in interpreting results. Nonetheless, some consistent results are beginning to emerge from this increasingly complex literature.

Several studies have sought to determine the impact of divergent hosts such as mosquitoes and birds, or host cell types, on WNV. These studies have included field (Jerzak et al., 2005), and *in vitro* (Ciota et al., 2007b, c) and *in vivo* (Jerzak et al., 2007) laboratory passage studies.

They showed that WNV displays high levels of within-host genetic diversity suggestive of quasispecies structure, and that WNV sequences from mosquitoes or mosquito cell culture were more genetically diverse than were sequences from vertebrates or vertebrate cell culture. *In vitro* passage studies showed that WNV can achieve much more significant adaptations to a mosquito cell line, as compared to a vertebrate cell line following sequential passage which bypassed the alternate cell type (Ciota et al., 2007c). These studies also demonstrated that no replicative cost is accrued in other hosts when WNV becomes highly adapted to a single cell type (Ciota et al., 2007a, c). Analysis of patterns of synonymous and nonsynonymous variation of *in vivo* passed WNV revealed strong purifying selection in vertebrate-passed WNV, but not in mosquito-passed WNV. Analysis of field-collected WNV from both hosts also suggests significant purifying selection. Collectively, the results from fitness studies and measures of natural selection suggest that WNV evolutionary dynamics are largely limited by reliance on birds for perpetuation in nature. The strength of purifying selection is set by birds, but infection of mosquitoes appears to provide the genetic diversity upon which natural selection acts.

5 Summary and Future Studies

Publications addressing WNV molecular epidemiology and genetic diversity have increased significantly in recent years. These studies have made several important contributions to the field. First, they have built upon extensive previous literature, demonstrating that WNV is a collection of widely geographically distributed genotypes that comprise some of the most successful arthropod-borne viruses known. This genetic diversity has facilitated molecular epidemiologic studies that have documented intercontinental movement of the virus and increased our understanding of the rate and mechanisms of an intracontinental spread. Second, important population parameters such as the mutation rate and demographic patterns of WNV have been described, and natural selective forces quantified. These studies have established that the evolutionary forces impacting WNV are similar to other arthropod-borne RNA viruses. Third, analysis of nucleotide sequences and the phenotypes of field collected viruses have identified coding sequences that are functionally important in facilitating both enzootic transmission of the virus and pathogenesis in vertebrates. Finally, fitness studies and analysis of intrahost genetic diversity have contributed to our understanding of how WNV interacts with hosts, and helped clarify the population genetic determinants

that facilitate perpetuation and adaptation of WNV in new environments.

Several important challenges remain. As mentioned earlier, the recent rapid increase in genetic data on WNV has led to the description of several proposed new WNV lineages. Antigenic studies using standardized immune reagents should be conducted to clarify the relationships of these newly proposed lineages to those that are widely accepted. Moreover, a reanalysis of the relationships among JEV serogroup viruses seems warranted. Second, the most obvious shortcoming of the extant literature on WNV genetic diversity and molecular epidemiology is an overarching reliance on partial genome sequences. While it does not appear that studies of sequence fragments are significantly biased in terms of ultimate conclusions, they may be significantly limited in their power to detect significant phylogenetic clusters, or any but the most obvious sequence motifs that may impact the phenotype. In addition, studies of selection are currently limited to examination of the most commonly sequenced genome region(s), usually the E glycoprotein. Studies of complete genomes should become the norm in the field, rather than the notable exception, with increasing availability of new technology for genome sequencing. Third, it is clear from all currently published molecular epidemiologic studies of WNV that there is abundant genetic diversity both within and between hosts. The significance of this diversity, however, is not at all clear. Nor is it clear how genetic diversity, replicative fitness and transmission phenotype are related. Studies addressing these issues, while rather complicated, are important given the success of WNV in the Americas. Finally, it is not clear whether results obtained from studies of WNV may be generalized to other arboviruses. It therefore seems important to broaden studies of WNV, which have been vigorous and productive, to include other agents that may emerge or re-emerge in the future such as Rift Valley fever and Chikungunya viruses. The complexity of the factors that impact evolution of mosquito-borne viruses needs to be addressed with carefully designed experiments in order to open new pathways to the eventual control of these pathogens.

References

Anderson RM, May RM (1982) Coevolution of hosts and parasites. Parasitology 85(Pt 2): 411–426

Anderson JF, Vossbrinck CR, Andreadis TG, Iton A, Beckwith WH, III, Mayo DR (2001) A phylogenetic approach to following West Nile virus in Connecticut. Proc Natl Acad Sci U S A 98:12885–12889

Asnis DS, Conetta R, Teixeira AA, Waldman G, Sampson BA (2000) The West Nile virus outbreak of 1999 in New York: the Flushing hospital experience. Clin Infect Dis 30:413–418

Bakonyi T, Hubalek Z, Rudolf I, Nowotny N (2005) Novel flavivirus or new lineage of West Nile virus, central Europe. Emerg Infect Dis 11:225–231

Bakonyi T, Ivanics E, Erdelyi K, Ursu K, Ferenczi E, Weissenbock H, Nowotny N (2006) Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg Infect Dis 12:618–623

Beasley DW, Davis CT, Guzman H, Vanlandingham DL, Travassos da Rosa APA, Parsons RE, Higgs S, Tesh RB, Barrett ADT (2003) Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. Virology 309:190–195

Beasley DW, Whiteman MC, Zhang S, Huang CY, Schneider BS, Smith DR, Gromowski GD, Higgs S, Kinney RM, Barrett AD (2005) Envelope protein glycosylation status influences mouse neuroinvasion phenotype of genetic lineage 1 West Nile virus strains. J Virol 79:8339–8347

Bertolotti L, Kitron U, Goldberg TL (2007) Diversity and evolution of West Nile virus in Illinois and The United States, 2002–2005. Virology 360(1):143–149

Bin H, Grossman Z, Pokamunski S, Malkinson M, Weiss L, Duvdevani P, Banet C, Weisman Y, Annis E, Gandaku D, Yahalom V, Hindyieh M, Shulman L, Mendelson E (2001) West Nile fever in Israel 1999–2000: from geese to humans. Ann N Y Acad Sci 951:127–142

Black WCIV, Moore CG (1996) Population biology as a tool for studying vector-borne diseases. In *The Biology of Disease Vectors*, pp. 393–416. Edited by Beaty BJ & Marquardt WC Niwot: University Press of Colorado.

Blackburn NK, Thompson DL, Jupp PG (1987) Antigenic relationship of West Nile strains by titre ratios calculated from cross-neutralization test results. Epidemiol Infect 99:551–557

Bondre VP, Jadi RS, Mishra AC, Yergolkar PN, Arankalle VA (2007) West Nile virus isolates from India: evidence for a distinct genetic lineage. J Gen Virol 88:875–884

Brault AC, Langevin SA, Bowen RA, Panella NA, Biggerstaff BJ, Miller BR, Nicholas K (2004) Differential virulence of West Nile strains for American crows. Emerg Infect Dis 10:2161–2168

Brault AC, Huang CY, Langevin SA, Kinney RM, Bowen RA, Ramey WN, Panella NA, Holmes EC, Powers AM, Miller BR (2007) A single positively selected West Nile viral mutation confers increased virogenesis in American crows. Nat Genet 39:1162–1166

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE (1989) Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol 70(Pt 1):37–43

Charrel RN, Brault AC, Gallian P, Lemasson JJ, Murgue B, Murri S, Pastorino B, Zeller H, de Chesse R, de Micco P, de Lamballerie, X (2003) Evolutionary relationship between Old World West Nile virus strains. Evidence for viral gene flow between Africa, the Middle East, and Europe. Virology 315:381–388

Ciota AT, Lovelace AO, Jones SA, Payne A, Kramer LD (2007a) Adaptation of two flaviviruses results in differences in genetic heterogeneity and virus adaptability. J Gen Virol 88:2398–2406

Ciota AT, Lovelace AO, Ngo KA, Le AN, Maffei JG, Franke MA, Payne AF, Jones SA, Kauffman EB, Kramer LD (2007b) Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture. Virology 357:165–174

Ciota AT, Ngo KA, Lovelace AO, Payne AF, Zhou Y, Shi P-Y, Kramer LD (2007c) Role of the mutant spectrum in adaptation and replication of West Nile virus. J Gen Virol 88:865–874

Davis CT, Beasley DW, Guzman H, Siirin M, Parsons RE, Tesh RB, Barrett AD (2004) Emergence of attenuated West Nile virus variants in Texas, 2003. Virology 330:342–350

Davis CT, Ebel GD, Lanciotti RS, Brault AC, Guzman H, Siirin M, Lambert A, Parsons RE, Beasley DW, Novak RJ, Elizondo-Quiroga D, Green EN, Young DS, Stark LM, Drebot MA, Artsob H, Tesh RB, Kramer LD, Barrett AD (2005) Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: Evidence for the emergence of a dominant genotype. Virology 342:252–265

DeMadrid AT, Porterfield JS (1974) The flaviviruses (group B arboviruses): a cross-neutralization study. J Gen Virol 23:91–96

Ebel GD, Dupuis AP II, Ngo KA, Nicholas DC, Kauffman EB, Jones SA, Young DM, Maffei JG, Shi P-Y, Bernard KA, Kramer LD (2001) Partial genetic characterization of West Nile virus strains, New York State. Emerg Infect Dis 7:650–653

Ebel GD, Carricaburu J, Young D, Bernard KA, Kramer LD (2004) Genetic and phenotypic variation of West Nile virus in New York, 2000–2003. Am J Trop Med Hyg 71:493–500

Ferenczi E, Racz G, Faludi G, Czegledi A, Mezey I, Berencsi G (2005) Natural foci of classical and emerging viral zoonoses. In: Berencsi G, Khan AS, Halouzka J (eds) Emerging Biological Threat. IOS, Amsterdam, pp 43–49

Glavits R, Ferenczi E, Ivanics E, Bakonyi T, Mato T, Zarka P, Palya V (2005) Co-occurrence of West Nile Fever and circovirus infection in a goose flock in Hungary. Avian Pathol 34:408–414

Goddard LB, Roth AE, Reisen WK, Scott TW (2003) Vertical transmission of West Nile virus by three California Culex (Diptera: Culicidae) species. J Med Entomol 40:743–746

Goto A, Yoshii K, Obara M, Ueki T, Mizutani T, Kariwa H, Takashima I (2005) Role of the N-linked glycans of the prM and E envelope proteins in tick-borne encephalitis virus particle secretion. Vaccine 23:3043–3052

Gould EA, Lamballerie X, Zanotto PM, Holmes EC (2003) Origins, evolution, and vector/host coadaptations within the genus *Flavivirus*. Adv Virus Res 59:277–314

Hammam HM, Clarke DH, Price WH (1965) Antigenic variation of West Nile virus in relation to geography. Am J Epidemiol 82:40–55

Hanna SL, Pierson TC, Sanchez MD, Ahmed AA, Murtadha MM, Doms RW (2005) N-linked glycosylation of west nile virus envelope proteins influences particle assembly and infectivity. J Virol 79:13262–13274

Hayes EB, Gubler DJ (2005) West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. Annu Rev Med 57:181–194

Herring BL, Bernardin F, Caglioti S, Stramer S, Tobler L, Andrews W, Cheng L, Rampersad S, Cameron C, Saldanha J, Busch MP, Delwart E (2007) Phylogenetic analysis of WNV in North American blood donors during the 2003–2004 epidemic seasons. Virology 363(1):220–228

Hubalek Z, Halouzka J (1999) West Nile fever – a reemerging mosquito-borne viral disease in Europe. [Review] [73 refs]. Emerg Infect Dis 5:643–650

Jerzak G, Bernard KA, Kramer LD, Ebel GD (2005) Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. J Gen Virol 86:2175–2183

Jerzak GV, Bernard K, Kramer LD, Shi PY, Ebel GD (2007) The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. Virology 360:469–476

Jia Y, Moudy RM, Dupuis AP, Ngo KA, Maffei JG, Jerzak GV, Franke MA, Kauffman EB, Kramer LD (2007) Characterization of a small plaque variant of West Nile virus isolated in New York in 2000. Virology 367:339–347

Kilpatrick AM, Meola MA, Moudy RM, Kramer L (2008) Temperature, viral genetics, and the transmission of West Nile virus by Culex mosquitoes. PLoS Pathog 27;4(6):e1000092

Komar N, Langevin S, Hinten S, Nemeth N, Edwards E, Hettler D, Davis B, Bowen R, Bunning M (2003) Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg Infect Dis 9:311–322

Kramer LD, Bernard KA (2001) West Nile virus infection in birds and mammals. Ann N Y Acad Sci 951:84–93

Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB (1998) Phylogeny of the genus Flavivirus. J Virol 72:73–83

Ladeau SL, Kilpatrick AM, Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. Nature 447:710–713

Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ (1999) Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 286:2333–2337

Lanciotti RS, Ebel GD, Deubel V, Kerst AJ, Murri S, Meyer R, Bowen M, McKinney N, Morrill WE, Crabtree MB, Kramer LD, Roehrig JT (2002) Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, europe, and the middle East. Virology 298:96–105

Langevin SA, Brault AC, Panella NA, Bowen RA, Komar N (2005) Variation in virulence of West Nile virus strains for house sparrows (Passer domesticus). Am J Trop Med Hyg 72:99–102

Lukacik G, Anand M, Shusas EJ, Howard JJ, Oliver J, Chen H, Backenson PB, Kauffman EB, Bernard KA, Kramer LD, White DJ (2006) West Nile virus surveillance in mosquitoes in New York State, 2000–2004. J Am Mosq Control Assoc 22:264–271

Lvov DK, Butenko AM, Gromashevsky VL, Kovtunov AI, Prilipov AG, Kinney R, Aristova VA, Dzharkenov AF, Samokhvalov EI, Savage HM, Shchelkanov MY, Galkina IV, Deryabin PG, Gubler DJ, Kulikova LN, Alkhovsky SK, Moskvina TM, Zlobina LV, Sadykova GK, Shatalov AG, Lvov DN, Usachev VE, Voronina AG (2004) West Nile virus and other zoonotic viruses in Russia: examples of emerging-reemerging situations. Arch Virol Suppl 18:85–96

MacKenzie JS, Lindsay MD, Coelen RJ, Broom AK, Hall RA, Smith DW (1994) Arboviruses causing human disease in the Australasian zoogeographic region. Arch Virol 136:447–467

Macdonald G (1961) Epidemiologic models in studies of vector-borne diseases. Public Health Rep 76:753–764

Malkinson M, Banet C (2002) The role of birds in the ecology of West Nile virus in Europe and Africa. Current Topics in Microbiology and Immunology (267), 309–322

Moudy RM, Meola MA, Morin LL, Ebel GD, Kramer LD (2007) A newly emergent genotype of west nile virus is transmitted earlier and more efficiently by culex mosquitoes. Am J Trop Med Hyg 77:365–370

Murgue B, Zeller H, Deubel V (2002) The ecology and epidemiology of West Nile virus in Africa, Europe and Asia. Japanese Encephalitis and West Nile Viruses 267:195–221

Nasci RS, Savage HM, White D, Miller JR, Cropp BC, Godsey MS, Kerst AJ, Bennett P, Gottfried K, Lanciotti RS (2001) West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. Emerg Infect Dis 7:742–744

Parreira R, Severino P, Freitas F, Piedade J, Almeida AP, Esteves A (2007) Two distinct introductions of the West Nile virus in Portugal disclosed by phylogenetic analysis of genomic sequences. Vector Borne Zoonotic Dis 7:344–352

Petersen LR, Roehrig JT (2001) West Nile virus: a reemerging global pathogen. Emerg Infect Dis 7:611–614

Pisano MR, Tolou H (1996) The topotype notion and the quasispecies concept: The yellow fever virus as example. Travaux Scientifiques des Chercheurs du Service de Sante des Armees 69–70

Rosen L (1987) Overwintering mechanisms of mosquito-borne arboviruses in temperate climates. Am J Trop Med Hyg 37:69S–76S

Ryman KD, Ledger TN, Weir RC, Schlesinger JJ, Barrett AD (1997) Yellow fever virus envelope protein has two discrete type-specific neutralizing epitopes. J Gen Virol 78(Pt 6):1353–1356

Shirato K, Miyoshi H, Goto A, Ako Y, Ueki T, Kariwa H, Takashima I (2004) Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. J Gen Virol 85:3637–3645

Smithburn KC, Hughes TP, Burke AW, Paul JH 1940 A neurotropic virus isolated from the blood of a native of Uganda. Am J Trop Med Hyg 20:471–473

Snappin KW, Holmes EC, Young DS, Bernard KA, Kramer LD, Ebel GD (2007) Declining Growth Rate of West Nile Virus in North America. J Virol 81:2531–2534

Varelas-Wesley I, Calisher CH (1982) Antigenic relationships of flaviviruses with undetermined arthropod-borne status. Am J Trop Med Hyg 31:1273–1284

Weaver SC (2006) Evolutionary influences in arboviral disease. Curr Top Microbiol Immunol 299:285–314

Zeller HG, Schuffenecker I (2004) West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas. Eur J Clin Microbiol Infect Dis 23:147–156

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