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A single positively selected West Nile viral mutation confers increased virogenesis in American crows

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Abstract

West Nile virus (WNV), first recognized in North America in 1999, has been responsible for the largest arboviral epiornitic and epidemic of human encephalitis in recorded history. Despite the well-described epidemiological patterns of WNV in North America, the basis for the emergence of WNV-associated avian pathology, particularly in the American crow (AMCR) sentinel species, and the large scale of the North American epidemic and epiornitic is uncertain. We report here that the introduction of a T249P amino acid substitution in the NS3 helicase (found in North American WNV) in a low-virulence strain was sufficient to generate a phenotype highly virulent to AMCRs. Furthermore, comparative sequence analyses of full-length WNV genomes demonstrated that the same site (NS3-249) was subject to adaptive evolution. These phenotypic and evolutionary results provide compelling evidence for the positive selection of a mutation encoding increased viremia potential and virulence in the AMCR sentinel bird species.

Before 1996, West Nile virus (WNV, *Flaviviridae: Flavivirus*), a mosquito-borne virus transmitted between avian hosts and mosquitoes in enzootic cycles, was associated with only

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AUTHOR CONTRIBUTIONS

This study was designed by A.C.B., R.M.K., A.M.P. and B.R.M. Mutant constructions were produced by C.Y.-H.H., R.M.K., S.A.L. and W.N.R. Avian infections were performed by R.A.B. and N.A.P. Viral titrations were performed by S.A.L., N.A.P., W.N.R. with assistance from A.M.P. Positive selection analyses were performed by E.C.H.

Accession codes

The following strains were included in the tree in Figure 1a, with isolate names, infected organisms and GenBank accession numbers for complete genome sequences in parentheses: Russia 1999 (Ast99-901, AY278441); New York 1999 (HNY99, human, AF202541); New Jersey 2000 (NJ2000.MQ5488, mosquito, AF404754); New York 1999 (NY99, equine, AF260967); New York 1999 (NY99, flamingo, AF196835); Mexico 2003 (TM171-03, crow, AY660002); Israel 1998 (IS-98.STD, stork, AF481864); Tunisia 1997 (Tunisia-97, human, AY268133); Italy 1998 (Italy1998, horse, AF404757); France 2000 (France-2000, horse, AY268132); Romania 1996 (RO57-50, mosquito, AF260969); Russia 1999 (Russia 1999, human, AF317203); Kenya 1998 (Kenya-98, mosquito, AY262283); China 2001 (China-01, unknown, AY490240); Egypt 1951 (Eg101, human, AF260968); Ethiopia 1976 (Eth76, bird, AY603654); Australia 1960 (MRM61C, mosquito, D00246); Uganda 1937 (WNFCG, human, M12294); South Africa 1989 (SPU116-89, human, EU068667); Czech Republic 1997 (97-103-Rabensburg, mosquito, AY765264)[28]; Russia 1998 (LEIV-Krnd88-190, tick, AY277251)[28].

URLs

The HYPHY package[27] is available through the Datamonkey website (http://www.datamonkey.org/).

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mild febrile infections during periodic epidemics in humans in Africa and the Middle East. A WNV encephalitis outbreak was identified in Romania in 1996, and in subsequent years (1997-2000 in Israel, 1997 in Tunisia, 1999 in Russia, 1999-2007 in the US and Canada)[1], WNV has been associated with neurological syndromes, including meningoence-phalitis and acute flaccid paralysis[2,3], becoming the largest source of arboviral encephalitis.

The Israeli and North American WNVs have been isolated from dead birds[1], indicating an association with avian virulence that has not been documented for other WNV strains. Widespread mortality of exotic birds in the Bronx Zoo and resident AMCRs (*Corvus brachyr-hynchos*)[4] was associated with human WNV encephalitis cases in 1999 (ref. 5). Experimental infection studies demonstrated low virulence of WNV strains from Australia and Kenya (KN-3829) in AMCRs[6]. In North America, AMCRs have been used as sentinels for WNV activity because of their high susceptibility to infection, their abundance in urban and periurban areas and the association of dead AMCRs with higher mosquito infection rates and human disease clusters[7].

AMCRs have a ubiquitous distribution throughout North America and are found in great numbers in almost all habitats, with the exception of the southwestern deserts. Their unique susceptibility to the North American strain of WNV, widespread distribution and close association with human habitation have made AMCRs the predomi-nant sentinel species for viral activity in North America. Another closely related species, Corvus ossifragus (the fish crow), has a reduced susceptibility to the North American strain of WNV[8]; however, differential susceptibilities of Corvus corone (the carrion crow) or *Corvus cornix* (the hooded crows) from Europe, Africa and Asia have not been assessed.

Viruses generated from infectious cDNAs of the North American NY99 strain 382-99 (ref. 9) (WN/IC-P991) and the Kenyan KN-3829 strain[10] (WN/IC-KEN) have corroborated the genetic basis for differential avian virulence of the parental strains[9]. A single amino acid residue within the NS3 helicase (Thr249 in the KN-3829 strain and Pro249 in the NY99 strain) was variable among different WNV isolates (Fig. 1a), with varying geographic distributions (Fig. 1b). We introduced point mutations by site-directed mutagenesis within the parental WNVs (WN/IC-KEN and WN/IC-P991) to generate recombinant viruses (WN/IC-KEN-NS3-T249P and WN/IC-P991-NS3-P249T, respectively). All viruses were derived from transfection of BHK-21 cells with *in vitro*-transcribed viral RNA and grew to 9.2-9.3 $\log_{10}$ plaque-forming units (PFU)/ml. We confirmed the genotype of all viruses by full-length sequencing.

The WN/IC-KEN strain did not induce a detectable viremia in AMCRs until 2 and 3 days post-infection (dpi) (Fig. 2a)[9]. Incontrast, the WN/IC-KEN-NS3-T249P virus rapidly induced a mean viremia of > 10,000 viral plaque-forming units (PFU) per ml of serum within 2 dpi. The WN/IC-KEN-NS3-T249P virus developed a mean viremic titer that was >10,000 times higher than that of the parental WN/IC-KEN strain at 3 dpi (Fig. 2a) and produced significantly ($P < 0.05$) higher mean viremic titers at 2-5 dpi. Mortality of AMCRs inoculated with WN/IC-KEN-NS3-T249P virus was 94%, whereas the WN/IC-KEN virus resulted in only 31% mortality ($P < 0.01$). Furthermore, AMCRs that succumbed to the WN/IC-KEN-NS3-T249P mutant had a mean day of death of 6.9 ± 0.5 ($n = 15$), whereas the average time of death for the virus lacking the substitution was 10.2 ± 1.5 dpi ($n = 5$) (Fig. 2b) ($P < 0.01$). The reciprocal mutant, WN/IC-P991-NS3-P249T, substantially attenuated the NY99 virus, resulting in a mean peak viremia of 3.5 $\log_{10}$ PFU/ml, compared with the 9.4 $\log_{10}$ PFU/ml mean peak titer of WN/IC-P991 ($P < 0.01$) (Fig. 2a). Viremic titers >10 $\log_{10}$ PFU/ml have been routinely measured in WNV NY99-infected AMCRs[6,11]. We did not observe any significant difference ($P > 0.05$) in either daily mean viremic titer for any of the 7 d, overall peak viremia, day of viremia onset or viremia duration between WN/IC-KEN and WN/IC-P991-NS3-P249T viruses.

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Additionally, we did not observe any significant difference in mortality between the WN/IC-KEN and WN/IC-P991-NS3-P249T viruses ($P = 0.6$). Mortality dropped from 100% (WN/IC-P991) to 12.5% for the WN/IC-P991-NS3-P249T mutant (Fig. 2b) ($P < 0.01$). Highly efficient intracellular RNA replication of WNVs containing the NS3-T249P substitution could mediate elevated viremia.

We performed phylogenetic analysis of 21 complete WNV genomes (Fig. 1a). WNV strains sampled globally in recent years, including strains from North America, formed a distinct clade (lineage I) with Kunjin viruses from Australia and two isolates sampled from China and Egypt[12] (Fig. 1a). By mapping the mutational changes at NS3-249 onto this phylogenetic tree, the variation at this site becomes evident. The two available lineage II isolates have a histidine at this site, whereas Kunjin viruses have an alanine residue (Fig. 1a,b). The evolution of the more recent strains of lineage I, including those associated with high mortality in corvids, is accompanied by a threonine-to-proline substitution at NS3-249. Most notably, T249P has evolved on at least three independent occasions within lineage I (and possibly four times, depending on the uncertain phylogenetic position of Russia 1999-Ast99-901): in the 1951 Egyptian isolate, in the 1996 Romanian and 1999 Russian isolates and within the Israeli/North American WNV clade (Fig. 1a)[13].

To determine the evolutionary pressures acting on the NS3-249 loci, we estimated the ratio of nonsynonymous (d_N) to synonymous (d_S) substitutions per site (where a value of $d_N/d_S > 1$ signifies positive selection) across the *NS3* gene for 21 isolates. Notably, amino acid residue 249 was the only site in NS3 found to be subject to positive selection ($d_N/d_S = \infty$; $P = 0.01$). Most of the other amino acid residues in NS3 were subject to relatively strong purifying selective constraints, as the mean d_N/d_S for the gene as a whole was only 0.030. Hence, the same amino acid residue that increased virulence in our AMCR animal model has also experienced a history adaptive evolution.

AMCRs are highly susceptible to fatal infection with WNV NY99, and their great sensitivity to this virus has made these birds particularly effective sentinels for WNV epidemic activity in North America[14] Experimental infection of AMCRs with the NY99 virus has consistently resulted in 100% mortality[6,11]. Although the numbers animals tested are small, this degree of susceptibility has also been observed experimentally in the ring-billed gull, the house finch and the related corvid, the black-billed magpie[11]. Blue jays, fish crows, common grackles and house sparrows show 33%-75% mortality after experimental challenge with the NY99 virus, whereas 17 other bird species resist the virus challenge[11]. Although North American birds show variable species-specific susceptibilities to WNV NY99 infection, the observed mortality in wild and captive birds is considered a unique hallmark of the WNV introduction into North America[15].

We have shown here that the NS3-249 site is a key virulence determinant of the NY99 WNV and that a threonine-to-proline substitution at this locus in the closely related KN-3829 virus sufficient to alter its relatively avirulent phenotype in AMCRs. The virulence phenotype of the WN/IC-KEN-NS3-T249P mutant closely approaches that of the NY99 strain in terms of viremia production and level of lethality for these birds. The phylogenetic pattern and subsequent positive selection analysis of multiple WNV genotypes indicates the independent emergence of three distinctive WNV genotypes containing this critical AMCR virulence determinant[13] As none of the WNVs containing threonine at the NS3-249 locus have been associated with virulence in birds, we suggest that selection for the NS3-T249P substitution has contributed significantly to the increased avian virulence, particularly in AMCRs, that has been observed for WNV in North America. Kunjin virus, which is less closely related to the NY99 virus and shows marked reductions viremia and virulence in both AMCRs[6] and house

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sparrows[16] contains an alanine at residue NS3-249, although other genetic differences probably contribute to the attenuated phenotype of Kunjin virus in birds.

Selection of WNV strains for increased replication in particular avian hosts could have indirectly resulted in higher mortality in AMCRs as a byproduct of this increased replicative efficacy. Replication of the viruses containing NS3-Pro249 consistently far surpassed published bird viremia thresholds required for infection of many North American mosquito vectors (~5 $\log_{10}$ PFU/ml serum)[17]. For the AMCRs infected with the NY99 strain, viremic titers sufficient for mosquito infection were evident from 2 dpi through death (which typically occurred at 5-6 dpi) (Fig. 2a)[6]. In contrast, individual AMCRs infected with the WN/IC-P991-NS3-P249T virus often failed to develop viremic titers sufficient to infect mosquito vectors and to be subsequently transmitted to new avian hosts[17]. Increased peridomestic enzootic transmission of NS3-Pro249 strains might subsequently result in increased human transmission. It remains to be determined if the high incidence of moderate and severe disease in humans in North America is due to a distinctive virulence potential of the introduced WNV strain or if it is the result of high levels of transmission in a naive host population.

Increased pathological manifestations in birds could also favor more efficient infection of mosquitoes. AMCRs with maximal WNV viremias are typically moribund and could be more readily fed upon by mosquitoes as a result of hyperthermia, decreased defensive behavior or increased respiration rates[9,18,19] that have previously been identified as attractants for host-seeking mosquitoes. Increased virulence could result in an abundance of moribund birds, presenting with elevated viremias in atypical microhabitats (such as on the ground, as opposed to at elevation) at times when they would have increased contact with bridge vectors.

The reproductive rate of a pathogen is defined as the number of infections that result from a single infected host. For many pathogens, virulence for the host correlates negatively with the reproductive rate if the transmission potential is reduced. Arthropod-borne infections, in which vectors serve to transfer the pathogen, are believed to tolerate higher virulence levels in hosts owing to diminished reliance on host mobility for direct transmission. Thus, models would predict that pathogenic phenotypes of certain arthropod-borne viruses, like WNV, with higher virulence costs could be an evolutionary trade-off with transmissibility, such that the level of virulence that maximizes the reproductive rate of the virus is favored by natural selection[20,21]. Here, we have provided independent evidence that (i) a single substitution at NS3-249 markedly alters the replication of a relatively attenuated strain of WNV in AMCRs, and (ii) the NS3-249 site shows evidence of positive selective pressure when multiple WNV genotypes are ana-lyzed. At face value, this suggests that corvid virulence *per se* is beneficial for WNV transmission in North America, presumably because it is also associated with increased viremia and hence potentially increased transmissibility by mosquito vectors that are of moderate or even low susceptibility to oral infection. However, because the NS3-T249P substitution was already present in the WNV genotype introduced to North America, it is likely that virulence in North American corvids has been a by-product of previous selective pressures for increased replication in unknown African or Asian avian species. Notably, in the 1951 Egyptian outbreak in which a WNV with the NS3-Pro249 substitution was isolated, virulence was also identified in experimentally infected hooded crows[22]. No avian virulence data exist for either the Romanian (1996) or Russian (1999) outbreaks; however, seropositive corvids were identified in Israel in 1999-2000 (ref. 23) and during the Egyptian outbreak in the early 1950s[22,24], indicating that different corvid species could have a reduced susceptibility to WNVs possessing the NS3-Pro249 substitution. Additional changes within the genetic backbones of these viruses could modulate avian virulence phenotypes.

Dissemination of the NS3-Pro249 genotype could be facilitated by increased global trade, as well as through increased replication in important hosts, such as specific avian species. Higher

viral replication in common bird species would probably promote rapid expansion of the epidemic and epizootic NS3-Pro249 genotypes, as has been seen since 1996; however, WNVs with alternative residues at this site could be adaptive for unknown enzootic requirements such as improved infection of specific enzootic mosquito vectors. For example, the Kenyan strain was isolated from a pool of male *Culex univittatus* mosquitoes. The failure of the NS3-T249P substitution to expand its geographic range in the 1950s could have been due to limited international commerce. Additional mutations in alternative WNV genomes could have a profound effect on host restriction regardless of the NS3-249 loci. Further study is needed on the interaction of this genetic residue with other viral proteins and the resulting effects on host susceptibility. Adaptive trade-offs for epizootic versus enzootic transmission could explain the persistence of alternative (NS3-249) enzootic genotypes. These studies highlight the potential for RNA viruses to rapidly adapt to changing environments, with selective mutations resulting in unforeseen profiles in epidemiology and disease presentation after introduction to alternative geographic regions.

METHODS

Rescue of recombinant WNV

To avoid incorporation of confounding cell culture-related genetic substitutions, virus from cells transfected with *in vitro*-transcribed RNA from the different viral mutants was harvested directly for use in experimental avian infections. Generation of infectious virus from the parental constructs has been described previously[9]. RNAwas extracted, cDNA was generated and sequencing[25] of the complete genomes of all mosaic viruses was performed to confirm the genetic identity of all viruses used for pathogenesis testing.

Virus inoculation

After-hatch year AMCRs were used for all pathogenesis testing of viral mutants. To control for the dose delivered and to assure the genetic identity of administered virus, infection was by subcutaneous needle inoculation rather than mosquito bite. We administered 100 μl of the diluted primary transfection culture containing 1,500 PFU/0.1 ml to the breast region of AMCRs. Viral doses approximated those estimated to be administered through the bite of an infected mosquito[17]. AMCRs from experimental and control groups were bled once daily by either jugular or brachial venipuncture with a 26-gauge syringe from 1-7 dpi and were monitored daily for signs of disease through 14 dpi. Samples were frozen at -80 °C until samples were titrated for infectious units, as previously described[6].

Viral titration

We assessed the titer of infectious virus by monitoring plaque formation on monolayers of Vero cells. Briefly, serial tenfold dilutions of AMCR serum were adsorbed to Vero cells, which were then overlaid with agarose as described previously[6]. PFU were counted at 3 dpi, and that number was multiplied by the dilution factor to determine viral titer per ml serum. The initial 1:10 dilution of serum, as well as the use of 250 μl of the lowest dilution, resulted in a limit of viral detection of 1.6 $\log_{10}$ PFU/ml serum. Inocula for all four viruses were back titrated by a plaque assay to confirm the uniformity of the administered doses.

Statistical analyses

Statistical analyses were performed on peak viremia, viremia duration, day of viremia onset and day of death. All analyses, with the exception of day of death, were performed by analyses of variance (ANOVA). Multiple comparisons were performed using Tukey's HSD adjustment for comparison of mean. As there were only two virus groups with birds that died, the comparisons of day of death were analyzed using a *t* test, using Welch's modification for

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unequal variances. Mortality and morbidity proportions were compared using Fisher's exact test.

Evolutionary analyses

We subjected 21 complete genomes of WNV (coding region only; alignment length of 10,302 nucleotides) to maximum likelihood (ML) phylogenetic analysis. Phylogenetic trees were estimated using the GTR+I+Γ_4 model of nucleotide substitution, available in the PAUP* package[26], with successive rounds of branch swapping. All parameter values are available from the authors on request. To determine the support for particular groupings on the phylogenetic tree, we performed a bootstrap resampling analysis using 1,000 replicate neighbor-joining trees estimated under the ML substitution model.

To determine the selection pressures acting on the *NS3* gene, we estimated the numbers of nonsynonymous (d_N) to synonymous (d_S) nucleotide changes per site; when $d_N > d_S$, this was indicative of positive selection. Site-specific (that is, codon-specific) selection pressures were determined using the fixed effects likelihood (FEL) method available in the HYPHY package[27] and accessed through the Datamonkey website. This method was run using the GTR substitution model on a neighbor-joining phylogenetic tree. Using this procedure, only codon 249 contained statistically significant evidence for positive selection.

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COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

References

1. Hayes EB. Epidemiology and transmission dynamics of west nile virus disease. Emerg. Infect. Dis 2005;11:1167–1173. [PubMed: 16102302]
2. Sejvar JJ. West Nile virus and "poliomyelitis". Neurology 2004;63:206–207. [PubMed: 15277609]
3. Solomon T, Ravi V. Acute flaccid paralysis caused by West Nile virus. Lancet Infect. Dis 2003;3:189–190. [PubMed: 12679260]
4. Steele, KE., et al. Vet. Pathol. 37. 2000. Pathology of fatal West Nile virus infections in native and exotic birds during the 1999 outbreak in New York City, New York; p. 208-224.
5. Weiss D, et al. Clinical findings of West Nile virus infection in hospitalized patients, New York and New Jersey, 2000. Emerg. Infect. Dis 2001;7:654–658. [PubMed: 11589170]
6. Brault AC, et al. Differential virulence of West Nile strains for American crows. Emerg. Infect. Dis 2004;10:2161–2168. [PubMed: 15663854]
7. Reisen WK, et al. Role of corvids in epidemiology of west Nile virus in southern California. J. Med. Entomol 2006;43:356–367. [PubMed: 16619622]
8. Langevin SA, Arroyo J, Monath TP, Komar N. Host-range restriction of chimeric yellow fever-West Nile vaccine in fish crows (*Corvus ossifragus*). Am. J. Trop. Med. Hyg 2003;69:78–80. [PubMed: 12932102]

9. Kinney RM, et al. Avian virulence and thermostable replication of the North American strain of West Nile virus. J. Gen. Virol 2006;87:3611–3622. [PubMed: 17098976]
10. Miller BR, et al. First field evidence for natural vertical transmission of West Nile virus in *Culex univittatus* complex mosquitoes from Rift Valley province, Kenya. Am. J. Trop. Med. Hyg 2000;62:240–246. [PubMed: 10813479]
11. Komar N, et al. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis 2003;9:311–322. [PubMed: 12643825]
12. Lanciotti RS, et al. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science 1999;286:2333–2337. [PubMed: 10600742]
13. Charrel RN, et al. Evolutionary relationship between Old World West Nile virus strains. Evidence for viral gene flow between Africa, the Middle East, and Europe. Virology 2003;315:381–388. [PubMed: 14585341]
14. Eidson M. "Neon needles" in a haystack: the advantages of passive surveillance for West Nile virus. Ann. NY Acad. Sci 2001;951:38–53. [PubMed: 11797803]
15. Kramer LD, Bernard KA. West Nile virus in the western hemisphere. Curr. Opin. Infect. Dis 2001;14:519–525. [PubMed: 11964871]
16. Langevin SA, Brault AC, Panella NA, Bowen RA, Komar N. Variation in virulence of West Nile virus strains for house sparrows (*Passer domesticus*). Am. J. Trop. Med. Hyg 2005;72:99–102. [PubMed: 15728874]
17. Reisen WK, Fang Y, Martinez VM. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J. Med. Entomol 2005;42:367–375. [PubMed: 15962789]
18. Day JF, Edman JD. Malaria renders mice susceptible to mosquito feeding when gametocytes are most infective. J. Parasitol 1983;69:163–170. [PubMed: 6338190]
19. Day JF, Edman JD. The importance of disease induced changes in mammalian body temperature to mosquito blood feeding. Comp. Biochem. Physiol. A 1984;77:447–452. [PubMed: 6142803]
20. Lipsitch M, Moxon ER. Virulence and transmissibility of pathogens: what is the relationship? Trends Microbiol 1997;5:31–37. [PubMed: 9025233]
21. Ewald PW. Host-parasite relations, vectors, and the evolution of disease severity. Annu. Rev. Ecol. Syst 1983;14:465–485.
22. Work TH, Hurlbut HS, Taylor RM. Indigenous wild birds of the Nile Delta as potential West Nile virus circulati ng reservoirs. Am. J. Trop. Med. Hyg 1955;4:872–888. [PubMed: 13259011]
23. Malkinson M, Banet C. The role of birds in the ecology of West Nile virus in Europe and Africa. Curr. Top. Microbiol. Immunol 2002;267:309–322. [PubMed: 12082995]
24. Work TH, Hurlbut HS, Taylor RM. Isolation of West Nile virus from hooded crow and rock pigeon in the Nile delta. Proc.Soc.Exp. Biol. Med 1953;84:719–722. [PubMed: 13134268]
25. Deardorff E, et al. Introductions of West Nile virus strains to Mexico. Emerg. Infect. Dis 2006;12:314–318. [PubMed: 16494762]
26. Swofford, D. Phylogenetics Analysis Using Parsimony; Version 4. Sinauer Associates; Sunderland, Massachusetts: 2003.
27. Pond SL, Frost SD. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 2005;21:2531–2533. [PubMed: 15713735]
28. Bakonyi T, Hubalek Z, Rudolf I, Nowotny N. Novel flavivirus or new lineage of West Nile virus, central Europe. Emerg. Infect. Dis 2005;11:225–231. [PubMed: 15752439]

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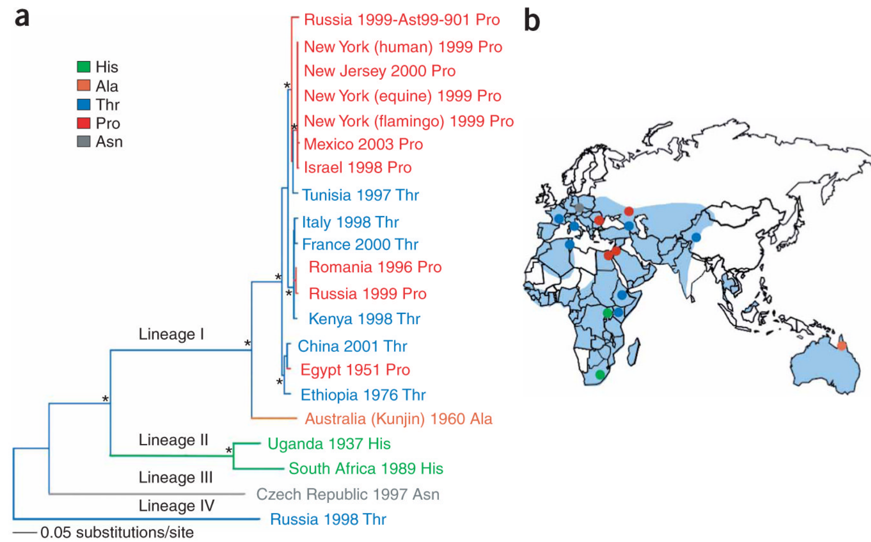

Figure 1.

Genetic relatedness and geographic distribution of WNV genotypes (**a**) Maximum likelihood phylogenetic tree of 21 complete genomes of WNV. Viruses are grouped according to the four described lineages of WNV, and the substituted amino acid at each NS3-249 site is indicated (and color coded). The most parsimonious reconstruction of the amino acid residue at internal nodes is also shown. The tree is midpoint rooted, and branch lengths are drawn to scale to indicate the number of substitutions per site. For details on the strains used in this tree, including isolate name, infected organism and GenBank accession number, see Methods. The asterisks represent nodes with bootstrap support values >95%. (**b**) Geographic distribution of WNVs in Africa, Europe and Asia (light blue). Colored dots indicate NS3-249 genotypic identity.

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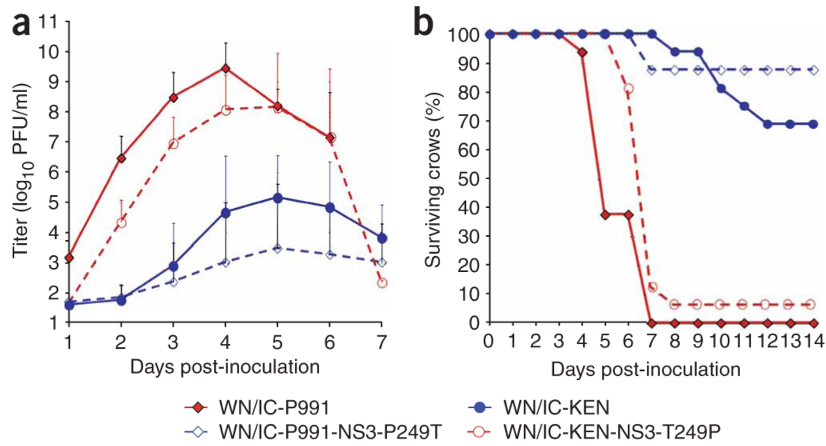

Figure 2.

Virulence of recombinant WNVs in AMCRs. (**a**) Viremia in AMCRs infected with WN/IC-P991 (*n* = 8), WN/IC-KEN (*n* = 16)[9], WN/IC-P991-NS3-P249T (*n* = 8) and WN/IC-KEN-NS3-T249P (*n* = 16) viruses. AMCRs were bled daily, and viral titers were determined by plaque titration on Vero cells with a detection limit of 1.6 log_{10} PFU/ml serum. Error bars represent s.d. (**b**) Survivorship of AMCRs inoculated with 1,500 PFU of WN/IC-P991 (*n* = 8) and WN/IC-KEN (*n* = 16) viruses[9] as well WN/IC-P991-NS3-P249T (*n* = 8) and WN/IC-KEN-NS3-T249P (*n* = 16) viruses. Parental viruses are designated by solid lines and symbols, and viral point mutants are designated by dashed lines and open symbols. Red lines and symbols represent viruses with the NS3-T249P substitution, and blue lines and symbols represent viruses with NS3-Thr249. Diamonds represent the WN/IC-P991 backbone, and circles represent the WN/IC-KEN backbone.