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Internally deleted WNV genomes isolated from exotic birds in New Mexico: Function in cells, mosquitoes, and mice

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ABSTRACT

Most RNA viruses exist in their hosts as a heterogeneous population of related variants. Due to error prone replication, mutants are constantly generated which may differ in individual fitness from the population as a whole. Here we characterize three WNV isolates that contain, along with full-length genomes, mutants with large internal deletions to structural and nonstructural protein-coding regions. The isolates were all obtained from lorikeets that died from WNV at the Rio Grande Zoo in Albuquerque, NM between 2005 and 2007. The deletions are approximately 2 kb, in frame, and result in the elimination of the complete envelope, and portions of the prM and NS-1 proteins. In Vero cell culture, these internally deleted WNV genomes function as defective interfering particles, reducing the production of full-length virus when introduced at high multiplicities of infection. In mosquitoes, the shortened WNV genomes reduced infection and dissemination rates, and virus titers overall, and were not detected in legs or salivary secretions at 14 or 21 days post-infection. In mice, inoculation with internally deleted genomes did not attenuate pathogenesis relative to full-length or infectious clone derived virus, and shortened genomes were not detected in mice at the time of death. These observations provide evidence that large deletions may occur within flavivirus populations more frequently than has generally been appreciated and suggest that they impact population phenotype minimally. Additionally, our findings suggest that highly similar mutants may frequently occur in particular vertebrate hosts.

Introduction

West Nile virus, (WNV), (*Flaviviridae: Flavivirus*) is a mosquito borne virus now endemic to the United States. It circulates between avian hosts and various mosquito vectors, mostly in the genus *Culex*. Since its first detection in the United States in 1999, WNV has dispersed throughout the western hemisphere (Artsob et al., 2009; Komar and Clark, 2006; Murray et al., 2010). WNV is an RNA virus, and as such accumulates mutations readily during replication because of the lack of a proof reading mechanism in the virus-encoded RNA dependent RNA polymerase, that can lead to the production of defective RNA genomes (Holland et al., 1982; Holmes, 2009). WNV and other arthropod-borne viruses (arboviruses) seem to have slower rates of evolution than single-host RNA viruses, which may be a result of evolutionary constraint imposed by the requirement for replication in both vertebrate and invertebrate cells (Jenkins et al., 2002). Supporting this, arbovirus populations in nature are subject to strong purifying selection (Weaver, 2006). Nonetheless, WNV and other arboviruses exist in mosquito and vertebrate hosts as a genetically heterogeneous mixture, with many individual mutants making up a genetically diverse population (Aaskov et al., 2006; Jerzak et al., 2005). Error-prone replication and consequent intrahost viral genetic diversity are, therefore, a central feature of the population biology of RNA viruses, including some arboviruses.

Defective interfering particles (DIPs) are subgenomic viral particles that arise during the course of viral infection, replicate through complementation with full-length homologous viruses, and have an inhibitory effect on virus growth (Thompson et al., 2009). DIPs have been detected after serial passage of the mosquito borne flaviviruses, WNV and Japanese encephalitis virus (JEV) in cell culture (Brinton, 1983, 2001; Debnath et al., 1991; Tsai et al., 2007; Yoon et al., 2006). Until recently, whether they exist in naturally acquired infections, and their role in shaping the outcome of virus–host interactions has been unclear. Importantly, isolates from patients with acute

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Dengue infections were recently shown to contain deletion mutants made up of the 5′ and 3′ untranslated regions of the genome (with the entire protein-coding sequence deleted), which behave *in vitro* as DIPs (Li et al., 2011). Isolates of another member of Flaviviridae, hepatitis C virus, containing large deletions to structural coding regions have been made from chronically infected individuals. Therefore, DIPs may occur in flavivirus infections more frequently than previously appreciated, and their presence within a virus population may significantly impact the outcome of infection in terms of transmission and pathogenesis.

Recently, we identified three WNV populations from lorikeets that died at the Rio Grande Zoo in two separate years that contain mutants with large internal deletions. We therefore sought to characterize these mutants and determine whether they function as WNV DIPs *in vitro*. We then determined the extent to which they influence transmission by mosquitoes and pathogenesis in mice to evaluate the possibility that they may contribute to the WNV perpetuation and/or disease outcomes in vertebrates. Our results suggest that naturally occurring WNV deletion mutants function as DIPs, and that these DIPs interfere with virus transmission by mosquitoes. However, the WNV DIPs we examined have surprisingly little impact on virus pathogenesis in mice.

Results

Identification of deletion mutants

RT-PCR targeting structural protein-coding regions of the WNV genome (Table 1: DH1F/DH1R) resulted in amplicons approximately 2 kb smaller than expected for samples from three birds that died of WNV infection (Fig. 1A). All three samples were taken from Rainbow Lorikeets (*Trichoglossus haematodus*) as confirmed by sequencing of the cytochrome oxidase (COI) gene. Isolates were made by inoculating Vero cells with kidney tissue homogenate of birds found dead at the Albuquerque Zoo in August of 2005 and 2007. Smaller genomes were confirmed directly using northern analysis of RNAs from one isolate (2774) infecting Vero cells, at 48 h post-infection and compared to infectious clone derived virus infected cells harvested 48 h post-infection. A probe to the NS-5 coding region annealed to a single band for RNA extracted from infectious clone derived virus infected cells but identified two bands in RNA extracted from cells infected with isolate 2774 (Fig. 1B), in contrast, a probe to the envelope coding region resulted in a single band produced for each RNA sample (Fig. 1C). Thus, shortened genomes could be detected directly with probes to NS-5 and appeared to make up around half of the viral RNA present within infected cells, but only full-length genomes were detected with envelope probe, as expected.

Table 1
Primer and probe sequences, DH = demi-hemi; numbers refer to location in full genome; qPCR MJ = quantitative reverse transcription polymerase chain reaction across mutant junction.

Name	Sequence
DH1F	ACTACTTCGGCTGTGTGAGCT
DH1R	ATGGGCCCTGGTTTTGTGTCT
10296rT7	CTA ATA CGA CTC ACT ATA GGG AGA TCC GAT GAT TGC TCT GAC TT
2666rT7	CTA ATA CGA CTC ACT ATA GGG AGA CGT CCT TCA CTG CTT CCC AGA
3′ end	AGATCCTGTGTTCTCGCACCA
qRT-PCR MJ F	ATC CGA GTG CTG GTG AGA CCA AAT
qRT-PCR MJ R	TTC CAA GGG AAG GTG ATG ATG ACG
qRT-PCR MJ probe	/56-FAM/GGA AAG AAC /ZEN/CTA AGC TTA GAA GTG GAG GA/3IABkFQ/

Genomic location of internal deletions

PCR amplicons from bird kidneys were cloned and sequenced to reveal large deletions within the structural and NS-1 coding regions. Deletions were in-frame and resulted in the loss of 3′ portions of prM, complete envelope, and 5′ portions of NS-1 encoding RNA (Fig. 1D). All eight clones sequenced from one kidney sample (2774) contained the same deletion, whereas the other two samples (3336, 3337) had two types of deletions present, labeled A and B in Fig. 1D. For 2774, the same deletion mutant was detected by cloning and sequencing of RT-PCR amplicons from the original kidney sample as well as Vero passages one and two. A probe was designed across the junction produced from the prM-NS-1 fusion that was detected in 2774, and was used in a qRT-PCR assay to directly detect the presence of this particular deletion mutant (Table 1). This assay estimated 4.6×10^3 mutant genome copies/0.1 ml in the original tissue sample of 2774, 2.7×10^5 mutant genome copies/0.1 ml in Vero passage 1 2774 and 2.6×10^6 mutant genome copies/0.1 ml in Vero passage 2 2774. Plaque assays estimated 2.4×10^4 pfu/0.1 ml in 2774 original kidney tissue, 4.7×10^4 pfu/0.1 ml in Vero passage one, and 3.7×10^6 pfu/0.1 ml in Vero passage 2.

Comparison of full genome sequences

Full genome sequencing of isolate 2774 revealed a predicted amino acid substitution, 2K-V9M, that had previously been detected in virus that was capable of bypassing superinfection exclusion (Zou et al., 2009b). Sequencing of the original sample to confirm this substitution showed a polymorphism at this site, both A and G appeared to be present at nucleotide (nt) position 6871 (determining 2K-V9 or 2K-M9, respectively). Sequencing of the other two isolates, 3336 and 3337, showed the same polymorphism present. To further characterize the presence of this polymorphism, RNA was extracted from individual plaques of 2774vp1, 2774vp3, 3336 (unpassed), and 3337 (unpassed) and subject to sequencing across the location of this mutation (nt.6871). The 6871G variant was more commonly detected and made up 7/10 plaques from 2774vp1, 2774vp3, and 3336, and 10/10 of the plaques from 3337. The 6871A variant was present as 3/10 plaques from 2774vp1, 2774vp3, and 3336, but not detected in any of the ten plaques from 3337, indicating it could be present only in the deletion mutant portion of this isolate. Nested PCR using primers designed to amplify solely internally deleted WNV genomes showed that 6871A was present for 2774. Bayesian analysis of partial genome sequences of NM isolates 3337 (NM05) and 2774 (NM07) did not group these two isolates together relative to other genomes sampled from the southwestern United States, indicating the mutation leading to this polymorphism may have arisen independently for each isolate (Fig. 2). Three of the consensus sequences shown in this phylogenetic tree (CA03, CA04, and NM07) had an M encoded at the 2K-9 site, whereas the remainder consensus sequences encode a V. However, it is currently not clear whether this variant may be present in other strains as a minority subpopulation.

Isolates inhibit production of full-length virus in Vero cells

To assess whether deletion mutants behave as defective interfering particles (DIPs), we evaluated full-length virus production from Vero cells infected over a range of multiplicity of infection (MOI) with isolate 2774 and with infectious clone derived WNV (WT). Full-length virus production was measured by plaque assay of cell culture supernatants after 3 days of growth in Vero cells. The full-length virus yield from each supernatant varied with virus isolate and viral dose, and these two factors had a significant interaction influencing virus production (Fig. 3A, $F=28.51$, $p=0.0014$). Supernatants from 2774 infected cells were tested by RNA extraction and quantitative RT-PCR to quantify the presence of deletion mutant