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Characterization of a small plaque variant of West Nile virus isolated in New York in 2000

Yongqing Jia [a], Robin M. Moudy [a], Alan P. Dupuis II [a], Kiet A. Ngo [a], Joseph G. Maffei [a], Greta V.S. Jerzak [a], Mary A. Franke [a], Elizabeth B. Kauffman [a], Laura D. Kramer [a,b,*]

[a] *The Arbovirus Laboratories, Wadsworth Center, New York State Department of Health, Slingerlands, NY 12159, USA*
[b] *Department of Biomedical Sciences, School of Public Health, State University of New York at Albany, Albany, NY 12201, USA*

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Abstract

A small-plaque variant (SP) of West Nile virus (WNV) was isolated in Vero cell culture from kidney tissue of an American crow collected in New York in 2000. The *in vitro* growth of the SP and parental (WT) strains was characterized in mammalian (Vero), avian (DF-1 and PDE), and mosquito (C6/36) cells. The SP variant replicated less efficiently than did the WT in Vero cells. In avian cells, SP growth was severely restricted at high temperatures, suggesting that the variant is temperature sensitive. In mosquito cells, growth of SP and WT was similar, but *in vivo* in *Culex pipiens* (L.) there were substantial differences. Relative to WT, SP exhibited reduced replication following intrathoracic inoculation and lower infection, dissemination, and transmission rates following oral infection. Analysis of the full length sequence of the SP variant identified sequence differences which led to only two amino acid substitutions relative to WT, prM P54S and NS2A V61A.

Keywords: West Nile virus; Small plaque variant; Wild-type; Temperature sensitivity; *Culex pipiens*; Vector competence

Introduction

West Nile virus (WNV) is an arthropod-borne virus belonging to the family *Flaviviridae*, genus *Flavivirus* that exists primarily in a mosquito–avian transmission cycle. WNV, which had been geographically limited to Africa, the Middle East, India, Australasia, and western and central Asia, was first introduced into the northeastern United States in 1999 (Lanciotti et al., 1999) and has since spread throughout the United States, into Canada, Mexico, the Caribbean, Latin America, and South America (Austin et al., 2004; Blitvich et al., 2003; Dupuis et al., 2005; Tachiiri et al., 2006). The single-stranded, positive-sense WNV genomic RNA is approximately 11 kb and encodes a single long polyprotein that is post-translationally processed by viral and host cellular proteases into three structural proteins and seven nonstructural proteins (Brinton, 2002). Comparisons of sequence data for full and partial genomes of WNV over the course of the virus' spread throughout the U.S. demonstrate that there has been minimal genetic change since its introduction in 1999 (Davis et al., 2004). Although WNV has remained relatively genetically homogeneous in the U.S., a new WNV genotype emerged late in the 2001 transmission season and has gradually established itself as the dominant genotype of WNV in the U.S. (Davis et al., 2005; Ebel et al., 2004). Concurrent with the emergence of this new genotype was the rapid spread of WNV across the U.S. leading to the largest recorded epidemic of arboviral encephalitis in the western hemisphere (CDC 2002). Several studies have confirmed the presence of distinct WNV genetic variants existing at temporal and geographical foci (Beasley et al., 2003; Davis et al., 2004, 2005); for example, small plaque isolates of WNV that exhibited attenuated properties *in vitro* and *in vivo* were collected in Texas in 2003 (Davis et al., 2004).

Small-plaque variants of other flaviviruses have been isolated in the laboratory. An isolate of dengue virus type 2 exhibited a mixed plaque phenotype in LLC-MK cells, and a small plaque subline selected from the mixed population in primary green monkey kidney cells was temperature sensitive

* Corresponding author. Arbovirus Laboratories, 5668 State Farm Road, Slingerlands, NY 12159, USA.
E-mail address: kramer@wadsworth.org (L.D. Kramer).

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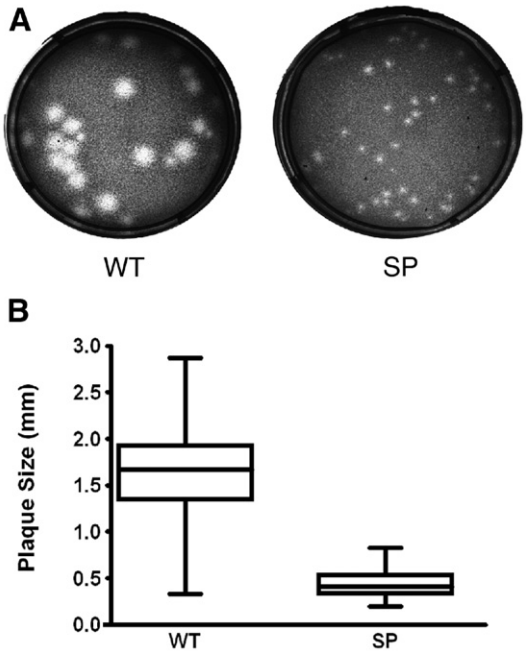

Fig. 1. Plaque morphology of wild-type (WT) and a small plaque (SP) variant WNV. Vero cells in 6-well plates were infected with Vero cell amplified WT or SP stock virus, and plaques were visualized 3 days post-infection after staining with neutral red. (A) Plaques were examined with a Zeiss Stemi 2000-C stereo microscope, and images were captured with a Zeiss Axiocam MRC digital camera. (B) Plaque sizes were measured using AxioVision 3.0 software (Zeiss, Germany). For each virus, 200 individual plaques were measured and analyzed by Graph Pad Prism (v.4.0). Bar within the box represents the median value, boxes extend from 25th to 75th percentile, and error bars represent highest and lowest values. Statistical analysis by *t*-test, $P<0.0001$.

(ts) in LLC-MK cells and attenuated in suckling mice (Eckels et al., 1976). A small plaque antigenic variant of Japanese encephalitis virus (JEV) was selected in Vero cell culture in the presence of a neutralizing monoclonal antibody, E3.3; this variant exhibited resistance to MAb3.3, but showed neurovirulence and neuroinvasiveness similar to the parental strain (Wu et al., 1997). Chemically mutated JEV, tick-borne encephalitis virus (TBEV), and dengue virus type 4 (DENV-4) have all manifested small plaque phenotypes, *ts*, and attenuated pathogenicity in mice (Blaney et al., 2001, 2002, 2003; Eastman and Blair, 1985; Hanley et al., 2002; Rumyantsev et al., 2006). Neuroblastoma cell-adapted yellow fever 17D virus exhibited a small plaque morphology, as well as defective cell penetration, poor cell-to-cell spread, and reduced growth efficiency, attributed to amino acid substitutions at positions 360 and 362 in the envelope protein (Chambers and Nickells, 2001; Nickells and Chambers, 2003; Vlaycheva and Chambers, 2002; Vlaycheva et al., 2004, 2005). An Ala to Pro single amino acid substitution in NS2A30 of WNV_{KUN} strain resulted in abortive viral replication with low titer, absence of detectable plaques, and high level INFα/β production in A549 (human alveolar basal epithelial) cells; however, in BHK cells, the strain's plaque size and replication efficiency were comparable to those of the wild-type (Liu et al., 2006).

Here we report a small-plaque (SP) variant selected from the mutant spectrum of WNV isolated from an American crow collected in New York in 2000. This variant was characterized *in vitro* in mammalian, avian, and mosquito cell lines, and *in vivo* in *Culex pipiens* (L.) mosquitoes. We demonstrate that this SP variant is attenuated both *in vitro* and *in vivo*.

Results

Isolation and characterization of the small-plaque variant

The plaque morphology of the SP variant of WNV, isolated as described in Materials and methods, was compared to the parental wild-type strain (WT). In Vero cells, at 72 h post infection (hpi), WT exhibited a mixed-plaque phenotype, producing both large and small plaques while the SP virus had uniformly small plaques (Fig. 1A). At this time point, SP virus plaques (mean: 0.44 mm in diameter, range: 0.20–0.83 mm) were significantly smaller than those of WT (mean: 1.62 mm, range: 0.34–2.87 mm; $P<0.001$, *t*-test; Fig. 1B). In this analysis, 100% of the SP population exhibited plaques less than 1.0 mm in diameter, whereas 91.5% of the WT plaques were larger than 1.0 mm.

In vitro growth kinetics

Growth of WT virus and the SP variant were compared in mammalian (Vero), avian (DF-1 and PDE), and mosquito (C6/36) cell lines. In Vero and DF-1 cells, at temperatures at or below 37 °C, the SP variant grew to significantly lower titers than did the WT ($P<0.01$, *t*-test; Fig. 2), but in PDE cells at 37 °C and 39 °C, the titers of WT and SP were similar. At higher temperatures, the growth kinetics of SP were dependent on the cell type. In avian cells, SP growth was severely restricted at temperatures at or above 41 °C, suggesting that the virus is ts in these cells. In Vero cells, although SP replicated more slowly than WT at 41 °C and 42.5 °C, the WT titers were equal to or lower than SP titers at later time points. This pattern could be due to high levels of WT viral replication resulting in increased cell mortality or deterioration of viable virus at high temperatures.

The small-plaque phenotype was retained following growth of the SP variant in all cell types at 30 °C, 34 °C, and 37 °C. However, in Vero and DF-1 cells at or above 41 °C, reversion to a mixed plaque phenotype was observed, with the proportion of plaques larger than 1.0 mm in diameter increasing with increasing temperature (data not shown). In PDE cells, growth

Fig. 2. Comparison of growth of wild-type (WT) and a small plaque (SP) variant WNV in three vertebrate cell lines. Vero, DF-1, and PDE cells in 6-well plates were infected with Vero cell amplified WT or SP stock virus in triplicate at an MOI of 0.01 pfu/cell, based on Vero cell titers. Both infection and incubation were carried out at the indicated temperatures. Virus was harvested from the medium at various time points after infection, and titers were determined by plaque assay on Vero cells. Symbols represent the mean titer and standard deviation for each time point. The limit of detection (LOD) was 0.7 $\log_{10}$pfu/0.1 ml of supernatant of infected cell cultures; in cases where the sample had no detectable virus, a titer of 0.65 $\log_{10}$pfu/0.1 ml was used for calculations.