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Brief Communication

West Nile virus adaptation to ixodid tick cells is associated with phenotypic trade-offs in primary hosts

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ABSTRACT

West Nile virus (WNV; *Flaviviridae, Flavivirus*) is the most geographically widespread arthropod-borne virus (arbovirus) in the world and is found in multiple ecologically distinct settings. Despite the likelihood of frequent exposure to novel hosts, studies evaluating the capacity and correlates of host range expansions or shifts of WNV and other arboviruses are generally lacking. We utilized experimental evolution of WNV in an *Amblyomma americanum* tick cell line to model an invertebrate host shift and evaluate the adaptive potential of WNV outside of its primary transmission cycle. Our results demonstrate that highly significant gains in replicative ability in ixodid tick cells are attainable for WNV but are also associated with widespread genetic change and significant phenotypic costs *in vitro*. Decreased fitness in primary hosts could represent a barrier to frequent exploitation of hard ticks by WNV in nature.

Introduction

Given the inherent requirement for host cycling of arthropod-borne viruses (arboviruses) evolution should theoretically favor generalists (Turner et al., 2010). *West Nile virus* (WNV; *Flaviviridae*, *Flavivirus*) has been particularly successful in a range of environments, resulting in a global distribution which is unprecedented among arboviruses. To date, WNV exists on all continents but Antarctica and can be classified into at least five distinct genetic lineages (May et al., 2011; Ciota and Kramer, 2013). This genetic diversity is likely attributed partly to stochastic change resulting from genetic isolation and drift, but also to adaptation to geographically distinct environments and transmission cycles. Although WNV is primarily maintained by *Culex spp.* mosquitoes and passerine birds, it has been isolated from over 75 mosquito and 300 avian species (Higgs et al., 2004; Marra et al., 2003; Hayes et al., 2005), as well as demonstrating competence in the laboratory for a range of taxonomically diverse hosts (Kramer et al., 2007). Although evolutionary theory would predict that host diversity may decrease the capacity for host-specific adaptation (Levins, 1968; Turner and Elena, 2000), the inherent generalism of WNV suggests it may be capable of continued niche expansion with relatively modest genetic change and cost in native hosts. The potential for host shifts is certainly substantial for a genetically diverse RNA pathogen such as WNV, which has few ecological barriers to host expansion. The tick burden on many highly competent avian hosts, for example, is often quite high, resulting in frequent tick exposure to WNV and therefore repeated adaptive opportunities (Hoogstraal, 1972). This is supported by the fact that WNV has frequently been isolated from many soft and hard tick species, including representatives from the *Argas*, *Ornithodoros*, *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Hyalomma* genera (Lwande et al., 2013; Moskvitina et al., 2008; Lawrie et al., 2004; Mumcuoglu et al., 2005; Hubalek and Halouzka, 1999). Entomological and genetic evidence suggests, in fact, that a lineage 2 WNV strain responsible for outbreaks in southern Russia and Romania may be maintained by *Hyalomma marginatum* ticks (Kolodziejek et al., 2014). Transmission by agrasid tick species has been demonstrated in the laboratory (Lawrie et al., 2004; Abbassy et al., 1993; Kokonova et al., 2013; Formosinho and Santos-Silva, 2006), yet similar studies with ixodid ticks failed to demonstrate competence (Anderson et al., 2003; Lawrie et al., 2004; Reisen et al., 2007).

Although many experimental evolution studies have assessed WNV adaptation and selective pressures using primary avian and mosquito hosts and experimental systems mimicking them (Ebel et al., 2011; Deardorff et al., 2011; Jerzak et al., 2008, 2007; Ciota et al., 2013, 2008, 2007a, 2007b, 2007c; Ciota and Kramer, 2010), studies to date have not adequately assessed the capacity and correlates of host shifts of WNV and other arboviruses. Here, we utilized passage of WNV in an ixodid tick cell line derived from

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Amblyomma americanum (AAE) to model an invertebrate host shift and subsequently evaluated the evolutionary capacity, genetic correlates and phenotypic costs for novel host adaptation. Our results provide insight into the adaptive potential and evolutionary consequences of WNV host expansion.

Results and discussion

In order to evaluate the extent of WNV adaptation to tick cells, as well as phenotypic consequences in alternate hosts, *in vitro* viral growth kinetics were determined on mammalian (Vero), avian (DF-1), mosquito (C6/36) and tick (AAE) cell lines following 20 passages on tick cell culture (AAE20). Results demonstrate increased replicative ability of WNV on AAE cells for both lineages 1 and 2 (L1, L2) following passage, with consistently higher titers measured for AAE20 strains relative to strains passaged once (AAE1; repeated measures ANOVA, $p=0.002$, tukey's post tests, $p<0.05$), and peak viral titers for AAE20 strains over 100-fold higher than AAE1 strains (Fig. 1). These results are consistent with the idea that WNV possesses a high capacity for adaptation to replication in novel invertebrate hosts. Although vector competence in natural systems is determined by multiple factors, gains in replicative fitness of this magnitude could conceivably increase the transmissibility of WNV by ixodid ticks. Similar studies recently completed with the closely related St. Louis encephalitis virus (SLEV; *Flaviviridae, Flavivirus*) demonstrated only modest adaptation to a *Dermacentor andersoni* line of tick cells (DAE), with fitness differences measured only after direct strain competition (Ciota et al., 2014). Although the use of different cell lines might partially explain these results, the superior adaptive potential of WNV relative to SLEV is consistent with differences in both levels of activity and global distribution (Reisen, 2003).

Previous studies with WNV suggest that host-specific adaptations are not necessarily associated with phenotypic costs in alternate hosts (Deardorff et al., 2011; Ciota et al., 2008, 2007b), and experimental evolution studies with other arboviruses together demonstrate that adaptation, although at times antagonistic (costly in alternate hosts), is also often generic (co or multi-adaptive) or neutral in other systems (reviewed in Ciota and Kramer, 2010). In contrast, results here demonstrate that adaptation to tick cells consistently results in highly significant decreases in replicative fitness in vertebrate and invertebrate cells. Specifically, consistently lower WNV titers were measured in DF-1, C6/36 and Vero cells for both lineages of AAE20 relative to AAE1 (repeated measures ANOVA, tukey's posttests, $p<0.01$; Fig. 1). In fact, since viral loads of AAE passaged strains are similar to input levels at 24 h in DF-1 cells, and there is no evidence of viral replication beyond 24 h, results suggest tick cell adaptation could result in an inability for WNV to propagate in avian cells. Since vertebrate and mosquito cells were grown at 37 °C and 28 °C, respectively, attenuated growth for AAE20 strains was also confirmed in Vero and C6/36 cells at 33 °C, the temperature at which the tick cells were maintained, demonstrating that adaptation cannot simply be attributed to temperature, but rather to more specific interactions with tick cells (Fig. 1). In addition to attenuated growth kinetics, impaired infection and/or cell to cell spread on mammalian cell culture were associated with AAE passage and adaptation. Decreases in both mean Vero plaque size (*t*-test, $p<0.05$; Fig. 2) and focus size (Fig. 3) were measured for AAE20 strains, with the larger costs measured with L2AAE20. Fluorescent focus assays were also completed on DAE tick cells, and results suggest that AAE adaptation, despite being costly in non-tick cells, is associated with increased infectivity in DAE cells and therefore generalizable to at least one other tick cell line (Fig. 3). Similarly, SLEV adaptation to DAE cells increased the capacity for replication in *Ixodes scapularis* cells (Ciota et al., 2014). Taken together, these data demonstrate that although WNV may be capable of high levels of co-adaptation with little phenotypic cost in its natural transmission cycle, more significant host shifts are likely to be detrimental to fitness in primary hosts, consistent with what has been predicated by evolutionary theory (Turner and Elena, 2000). Although *in vitro* systems are certainly not precise representatives of natural hosts, this provides a possible explanation for the fact that WNV has not readily exploited hard ticks in nature, despite the likelihood of frequent encounters.

Full-genome sequencing was completed in order to determine the genetic correlates of tick cell adaptation. A total of 9 and 11 substitutions were identified in WNV L1AAE20 and L2AAE20,

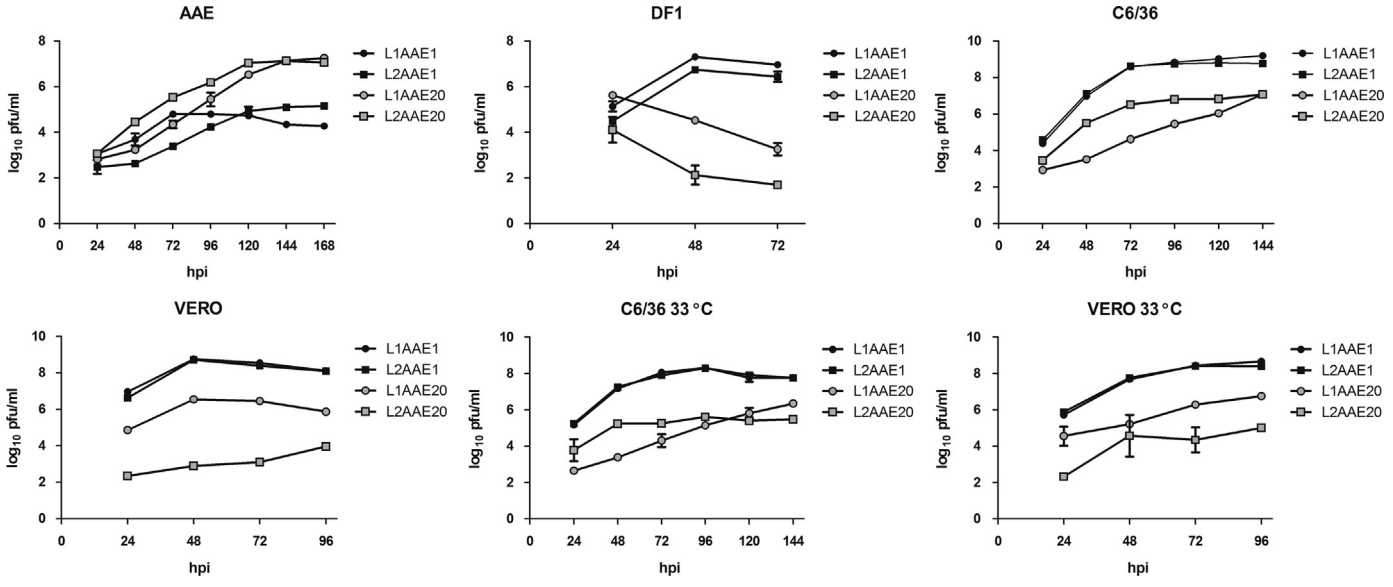

Fig. 1. Alterations to viral growth kinetics resulting from WNV passage on AAE tick cells. Viral growth was evaluated following infection at a MOI of 0.1 pfu/cell in tick (AAE), avian (DF-1), mosquito (C6/36) and mammalian (Vero) cell culture for 2 lineages (L) after 1 (AAE1) or 20 (AAE20) passages at 33 °C (AAE), 37 °C (DF-1, Vero) or 28 °C (C6/36), unless otherwise designated. Significantly different kinetics were measured for WNV AAE20 strains relative to AAE1 strains in all assays (repeated measures ANOVA, $p<0.001$) such that consistently higher WNV titers were measured on AAE cells f and consistently lower WNV titers were measured in DF-1, C6/36 and Vero cells for both AAE20L1 and AAE20L2 (tukey's posttests, $p<0.01$).

respectively, including six nonsynonomous changes in each (Table 1). Although this represents relatively minor change in the context of the entire genome, it stands in contrast to previous studies demonstrating that adaptation to mosquito cells is associated with little to no consensus change (Ciota et al., 2007b, 2007c). It is not surprising that a larger host jump requires more significant genetic alteration, and this is also consistent with the fact that larger phenotypic costs were identified. Surprisingly, not a single substitution is shared among adapted lineages, demonstrating that there are multiple, distinct adaptive landscapes for individual hosts which may require a series of epistatic interactions. This also suggests that evolutionary and adaptive potential are likely to be strain-specific. The fact that 4 of 12 amino acid substitutions were identified in a less than 300 base pair region of the NS4A gene suggests that this may be an important region in host range determination. Despite its relatively small size (150 AA), the NS4A is a multifunctional and highly interactive protein implicated in flavivirus replication, membrane formation and cell/immune signaling (Ambrose and Mackenzie 2011a, 2011b; Roosendaal et al., 2006). This gene has often been associated with host-specific WNV adaptation (Ciota et al., 2007a; Ciota et al., 2008; Jerzak et al., 2008) and it is plausible that this is a result of cell-specific immune responses. Yet, given the numerous functions of NS4A and the spacing of mutations across a ~100 AA region, it is difficult to confidently speculate on mechanisms of adaptation. In addition, with substitutions spread across 5 different genes it is possible that complex interactions among multiple proteins are responsible for the adaptive phenotype. What is conspicuously missing from L1AAE20 is mutation in the WNV envelope gene, suggesting that inefficient viral fusion and receptor binding does not necessarily constrain WNV fitness in tick cells. Future studies utilizing reverse genetics will help identify the correlates and mechanisms of WNV host range shifts.

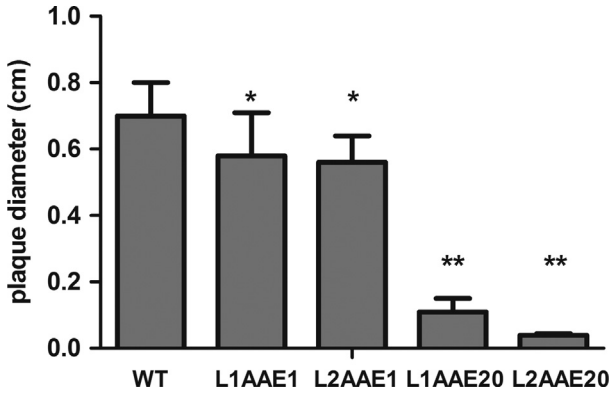

Fig. 2. Decreased WNV Vero plaque size is associated with tick cell passage. Plaques were randomly selected for measurement following standard plaque titration before (WT) or after 1 (AAE1) or 20 (AAE20) passages. The graph depicts means of 15 +/− SD and statistical significant differences relative to WNV WT (*t*-test) are denoted by * ($p < 0.05$) or ** ($p < 0.001$).

Table 1
West Nile virus nucleotide (NT) and amino acid (AA) substitutions identified in two lineages (L1 and L2) following 20 passages in AAE tick cells (AAE20).

WNV L1AAE20			WNV L2AAE20	
NT change	AA change	gene	NT change	AA change
C716T	T207I	pr		
		pr	G737A	R214Q
		M	T246C	L217P
G756A		M		
C763T	R214W	M		
		ENV	C2063T	T656M
		ENV	T2073C	
		ENV	G2134A	E680K
A2747C	Q884P	NS1		
		NS1	T3093C	
		NS4A	T4194C	
T6482C	L2129P	NS4A		
C6511T	H2139Y	NS4A		
T6644C	L2183S	NS4A		
		NS4A	A6776G	K2227R
		NS4B	C7078T	
T8349C		Mtase		
T8364C		Mtase		
		Mtase	T8373C	
		RdRp	A9443G	D3116G

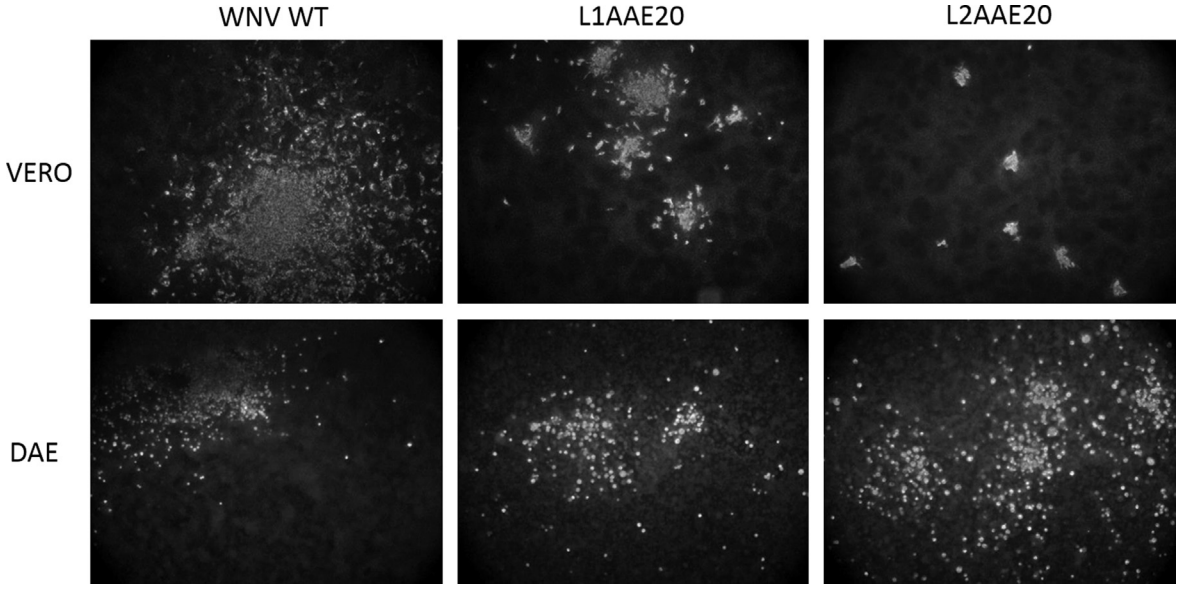

Fig. 3. Increased WNV infection and spread on tick cells is associated with decreased WNV infection and spread on vertebrate cells. Fluorescent focus assays were performed on mammalian (Vero) or tick (DAE) cells before (WT) or after (AAE20) tick cell passage. WNV antibody was labeled with FITC conjugate and cells were visualized with fluorescent microscopy at 48 h (Vero) or 72 h (DAE) post infection.

Conclusions

Pathogens will be unable to exploit new hosts if (i) ecological or environmental barriers prevent frequent contact, (ii) evolutionary (genetic) barriers prevent sufficient gains in fitness (infectivity, replication and/or transmissibility), or (iii) adaption to a novel host is sufficiently costly in primary hosts. Phenotypic costs are particularly important for arboviruses given the requirement for amplification in alternate hosts. Since ecological barriers for WNV adaptation to ticks do not exist, we exploited experimental evolution in AAE ticks cells to gain insight into both the adaptive potential of WNV in ixodid tick cells and the phenotypic consequences of such adaption in primary host cells. Our results demonstrate that WNV has a high capacity for adaptation to AAE tick cells, with unique, widespread genetic change resulting in substantial increases in viral replication in two separate lineages following passage, yet also that increased replicative ability in tick cells is associated with significantly impaired viral replication and/or spread in mosquito, avian and mammalian cells. These phenotypic costs could function to prevent exploitation of hard ticks by WNV in nature.

Methods

Cells and media

AAE cells [kindly provided by Timothy Kurtti, Univ. of Minnesota (Munderloh et al., 2003)] were grown in L-15B medium, pH 7.0, containing 5% tryptose phosphate broth, 10% fetal bovine serum, 0.1% bovine lipoprotein cholesterol concentrate and maintained at 33 °C in 5% CO_2. African green monkey kidney cells (Vero, ATCC #CCL-81) and Chicken embryo fibroblast cells (DF-1, ATCC #CRL-12203) were grown in minimal essential medium (MEM, Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 U/ml of penicillin, and 100 ug/ml of streptomycin and maintained at 37 °C in 5% CO_2. *Aedes albopictus* cells (C6/36, ATCC #CRL-1660) were grown in MEM supplemened with 10% FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 100 U/ml of penicillin, and 100 ug/ml of streptomycin and maintained at 28 °C in 5% CO_2.

Virus passaging

WNV 3356.1.1.1 (WT), a biological clone derived from 3 rounds of plaque purification of the WNV NY99 isolate NY003356 (Ebel et al., 2001; Ciota et al., 2007b), was used for passaging. Confluent monolayers of AAE cells were infected at a MOI of 1.0 pfu/cell for each passage. Supernatant was harvested for each of 2 lineages (L1, L2) at 9 days post infection (pi), quantified by plaque titration on Vero cell culture (Payne et al., 2006), and diluted in order to maintain MOI prior to subsequent passage. Phenotypic and genetic characterization were completed on strains following 20 AAE passages (AAE20) and compared to either WNV WT or single passage strains (AAE1).

Viral growth kinetics and infectivity

Confluent monolayers of mammalian (Vero), avian (DF-1), mosquito (C6/36) or tick (AAE) lines were infected with virus, in duplicate, using 6-well plates, at a MOI of 0.1 pfu/cell by standard protocol (Ciota et al., 2007b). Briefly, following a 1 h viral absorption period, the inoculum was removed, cells were gently washed then overlaid with 2 ml of appropriate maintenance media. Absorption and growth proceeded at standard temperatures for each cell line except where indicated (i.e. Vero and C6/36 at 33 °C). 100 ul samples of supernatant were taken daily for a total of 3–7 days based on previous kinetics, diluted 1:10 in media containing 20% FBS, and stored at −80 °C. Titrations were performed in duplicate, by plaque assay on Vero cells and mean titers for each time point were calculated and compared using standard two-way *t*-tests. Plaque diameters were measured using a Zeiss microscope and compared by standard *t*-tests (GraphPad Prism, Version 5.0).

Fluorescent focus assay were performed as previously described (Payne et al., 2006; Ciota et al., 2014). Briefly, confluent 8-well chamber slides (Nalge Nunc International) were inoculated with ten-fold serial dilutions of virus in a final volume of 0.05 ml; virus was adsorbed to the cells for 1 hr at 37 °C (Vero) or 33 °C (DAE) and overlaid with MEM, 5%FBS with 0.8% carboxy methyl cellulose (CMC, ICN Biomedicals; Vero) or L-15B (DAE). After 48 (Vero) or 72 (DAE) hrs cells were washed with cold phosphate-buffered saline (PBS). Fixation was performed for 10 min with ice-cold absolute methanol (Sigma-Aldrich) and slides were placed in a moist chamber in WNV antibody (WNV MAb 5H10, Bioreliance) diluted 1:200 in PBS containing 0.2% BSA (PBS-BSA). Antibody-labeled cells were detected with a secondary antibody conjugated to FITC (KPL) diluted 1:50 in PBS-BSA. Cells were mounted in anti-fading medium (Vector Laboratories) and visualized with a Zeiss Axiovert 25 microscope, equipped with a Fluar 10 × objective, and FITC filter. Images were photographed with a Zeiss Axiocam MRC digital camera and Axiovision software was used for foci measurement.

Virus sequencing

Full-genome sequencing was completed as described elsewhere (Ciota et al., 2007b) using 9 overlapping primer sets (sequences available upon request). Briefly, RNA was extracted from cell culture supernatant and subjected to reverse transcription (RT) and polymerase chain reactions (PCR) using the SuperScript III one-step RT-PCR kit (Life technologies) and products were concentrated using Zymo-5 DNA spin columns (Zymo Research). Sequencing was completed at the Wadsworth Center Applied Genomics Technology Core on an ABI 3100 or 3700 automated sequencer (Applied Biosystems). Sequences were compiled and edited using the SeqMan module of the DNAStar software package (DNAStar) with a minimum of two-fold redundancy throughout the genome.

Acknowledgments

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