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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,

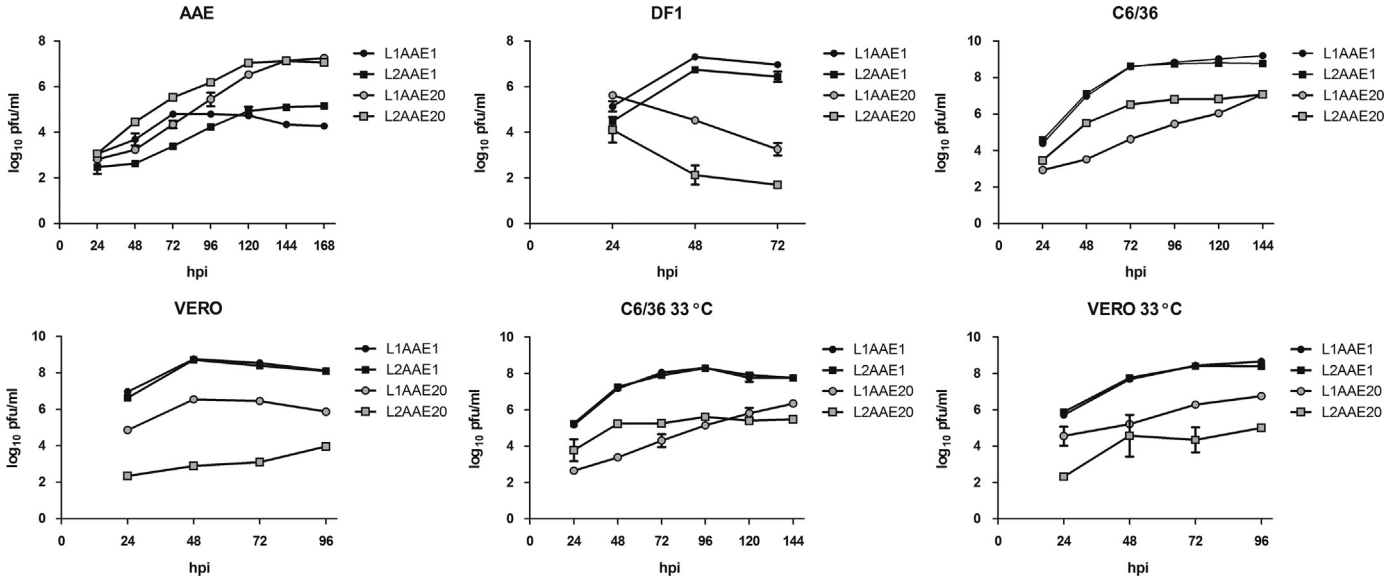

Fig. 1. Alterations to viral growth kinetics resulting from WNV passage on AAE tick cells. Viral growth was evaluated following infection at a MOI of 0.1 pfu/cell in tick (AAE), avian (DF-1), mosquito (C6/36) and mammalian (Vero) cell culture for 2 lineages (L) after 1 (AAE1) or 20 (AAE20) passages at 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, $p<0.01$).