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Adaptation of two flaviviruses results in differences in genetic heterogeneity and virus adaptability

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West Nile virus (WNV) is a mosquito-borne flavivirus that was first introduced into the USA in the New York City area in 1999. Since its introduction, WNV has steadily increased both its host and geographical ranges. Outbreaks of the closely related flavivirus, St. Louis encephalitis virus (SLEV), occur in the USA periodically, but levels of activity and host range are more restricted than those of WNV. Understanding the selective pressures that drive arbovirus adaptation and evolution in their disparate mosquito and avian hosts is crucial to predicting their ability to persist and re-emerge. Here, we evaluated the *in vivo* phenotypes of mosquito cell-adapted WNV and SLEV. Results indicated that *in vitro* adaptations did not translate to *in vivo* adaptations for either virus, yet SLEV displayed attenuated growth in both mosquitoes and chickens, while WNV generally did not. *In vitro* growth analyses also indicated that WNV adaptations could be generalized to cell cultures derived from other mosquito species, while SLEV could not. Analysis of genetic diversity for passaged SLEV revealed a highly homogeneous population that differed significantly from previous results of high levels of diversity in WNV. We hypothesize that this difference in genetic diversity is directly related to the viruses' success in new and changing environments in the laboratory and, that differences in a viruses' ability to produce and maintain heterogeneous populations in nature may in some instances explain the variable levels of success seen among arboviruses.

INTRODUCTION

West Nile virus (WNV) is a mosquito-borne flavivirus that was first introduced into the USA in the New York City area in 1999. Since its introduction, WNV has steadily increased both its host and geographical range, spreading across the USA and into Canada, Mexico, and Central and South America (Lanciotti *et al.*, 1999; Dupuis *et al.*, 2003; Dupuis *et al.*, 2005; Austin *et al.*, 2004; Cruz *et al.*, 2005; Elizondo-Quiroga, 2005; Granwehr *et al.*, 2004; Morales *et al.*, 2006). Worldwide, WNV has infected over 75 species of mosquitoes (Higgs *et al.*, 2004) and over 300 species of birds (Marra *et al.*, 2003). St. Louis encephalitis virus (SLEV) is a flavivirus that is highly genetically and antigenically similar to WNV (Monath & Heinz, 1996). Outbreaks of SLEV occur in the USA periodically (Day & Stark, 2000; Chandler *et al.*, 2001; Reisen, 2003), but levels of activity and host range have never reached those of WNV (http://www.cdc.gov/ncidod/dvbid/arbor). Evolutionary pressures on these viruses are exerted almost exclusively by the ornithophilic mosquito and avian hosts that sustain their transmission cycles (Kramer & Bernard, 2001). Understanding the selective pressures that drive arbovirus adaptation and evolution in these disparate environments is crucial to predicting their ability to persist and re-emerge.

Many studies have used cell culture models to characterize arbovirus adaptation to diverse environments and to assess the level of evolutionary compromise resulting from the cycling of replication in disparate hosts (Domingo & Holland, 1997; Cooper & Scott, 2001; Holland *et al.*, 1991; Novella *et al.*, 1999a, b; Weaver *et al.*, 1999; Ciota *et al.*, 2007a; Chen *et al.*, 2003; Zarate & Novella, 2004). Although previous studies with flaviviruses have shown that cell culture passage can lead to attenuation in various *in vivo* systems (Miller & Mitchell, 1986; Barrett *et al.*, 1990; Halstead *et al.*, 1984; Hearn, *et al.*, 1966; Eckels *et al.*, 1984; Goto *et al.*, 2003), to our knowledge, no studies have specifically evaluated how adaptation to an insect cellular environment is reflected in both corresponding and disparate *in vivo* hosts. In a previous study, we measured relative fitness and replicative ability *in vitro*, and assessed genetic alterations of both WNV and SLEV following serial passage in mosquito cell culture (Ciota *et al.*, 2007a). Our results indicated significant cell-specific adaptation to these cells resulting from serial passage. The magnitude and rate of adaptation were greater with SLEV compared with WNV. We also reported that serial passage of WNV in mosquito cells resulted in highly genetically diverse populations, and that aspects of the mutant spectrum are likely responsible for generating the adaptive phenotype of this virus (Ciota *et al.*, 2007b). Here, using the same passage series, we sought to clarify whether these *in vitro* adaptations translated to phenotypic changes *in vivo* and, subsequently, how useful these types of cell culture

models are in identifying alterations potentially important in natural arbovirus adaptation and evolution. Specifically, we used virus populations before and after *Aedes albopictus* mosquito (C6/36) cell passage to measure vector competence in *Culex pipiens* mosquitoes following blood feeding, virus growth in *Cx. pipiens* following inoculation, and viraemia in chickens following inoculation. We also measured the replicative phenotypes of these virus populations in a cell line derived from *Anopheles gambiae* in order to evaluate the specificity of the virus adaptations.

The genetic diversity generated with mosquito cell passaged SLEV populations was evaluated in order to assess possible differences in the size of the mutant spectra of this virus and WNV populations. Adaptability of virus populations and success in new and dynamic environments are clear benefits of diverse quasispecies structures. Studies with foot-and-mouth disease virus (FMDV) (Arias *et al.*, 2004; Ruiz-Jarabo *et al.*, 2000) and human immunodeficiency virus (Briones *et al.*, 2006) have identified the presence of molecular memory of passage history in intrahost populations. A study with the multiple plant viruses has shown a correlation between host range and the size of the mutant spectrum (Schneider & Roossinck, 2000). We hypothesize that differences in the plasticity of SLEV and WNV populations may be a direct result of each virus' ability to produce and maintain sufficiently adaptable mutant spectra. These differences could also explain differences in adaptation observed in previous studies and, more broadly, have implications for understanding the epidemiological patterns of arbovirus activity in nature.

METHODS

Mosquitoes.

Cx. pipiens mosquitoes were originally collected in Pennsylvania in 2004 and colonized at the Wadsworth Center insectary. Mosquitoes were reared in environmental chambers at 27 °C, 70% relative humidity with a photoperiod of 16:8 (light:dark) h. Adult female mosquitoes used for experiments were held in mesh top 3.8 L paper cartons and provided cotton pads soaked in 10% sucrose *ad libitum*. Mosquitoes were held for 4–7 days before being transferred to 0.6 L cups for experimental infections in the BSL-3 facility.

Chickens.

Pathogen-free chicken eggs (*Gallus gallus*) were obtained from Sunrise Farms (Catskill, NY) and hatched in an incubator (G.Q.C) at the Arbovirus laboratories. Newborn chickens were transferred 3–12 h post-hatching to the BSL-3 animal facility in preparation for virus

inoculations. Chickens were separated by experimental group and housed in metal cages with individual light sources and daily fresh food, water and resting pads.

Viruses.

Biological clones of WNV (3356.1.1.1) and SLEV Kern (217.3.1.1) were produced by plaque purification and amplification on Vero cells as described previously (Ciota *et al.*, 2007a). Sequential passage of viruses was completed at an m.o.i. of 0.1 p.f.u. per cell as described previously (Ciota *et al.*, 2007a) for WNV passaged 39 times in C6/36 cells (WNV CP39), and SLEV passaged 6, 10 or 40 times in C6/36 cells (SLEV CP6, SLEV CP10 and SLEV CP40, respectively). Multiple aliquots of all viral stocks were stored frozen at –80 °C.

Infectious dose-50 (ID_{50}) and virus growth curve assays in mosquitoes.

Female *Cx. pipiens* were infected by intrathoracic inoculation (Rosen & Gubler, 1974) for both determination of ID_{50} (dose at which 50% of mosquitoes become infected) and growth of individual virus strains. The ID_{50} for each WNV strain was determined by inoculation of 10 mosquitoes per dilution using 10-fold increasing concentrations of virus from 0.1 p.f.u., and screening for infection by plaque assay on Vero cell culture at 7 days post-inoculation (p.i.). Calculations of ID_{50} were done using the Reed–Muench formula. Inoculations for growth curve assays were done with 10–20 times the ID_{50} and viral titre was determined for 5–10 mosquitoes per time-point harvested 1–7, 14 and 21 days p.i. For both assays, mosquitoes were individually frozen at –80 °C at appropriate time points in 2.0 ml microcentrifuge tubes filled with 1 ml mosquito diluent [MD; 20% heat-inactivated fetal bovine serum (FBS) in Dulbecco's PBS plus 50 μg penicillin/streptomycin ml^{-1}, 50 μg gentamicin ml^{-1} and 2.5 μg Fungizone ml^{-1}] plus one 5 mm metal bead (Daisy). Samples were thawed for testing and homogenized for 30 s at 20 Hz in a Mixer Mill MM301 (Retsch). Debris was then pelleted by centrifugation at 6000 rcf for 5 min and titrated or screened by plaque assay in duplicate on Vero cells as described previously (Payne *et al.*, 2006).

Vector competence.

Infection, dissemination and transmission rates were determined as previously described (Ebel *et al.*, 2005). Briefly, 7-day-old female *Cx. pipiens* were deprived of sucrose for 48 h and water for 24 h, and then offered a pledget saturated with a 1:10 mixture of the appropriate virus:defibrinated goose blood (HemaResources) with a 2.5% final sucrose concentration. After 1 h of exposure to the bloodmeal, mosquitoes were sedated with CO_2 and fully engorged mosquitoes were transferred to 0.6 L cartons and reserved for experimental testing. On days 5, 7, 9 and 14 post-feeding, 35–50 mosquitoes from each sample group were sedated and legs were removed and placed in 1 ml MD. Capillaries

filled with FBS plus 50% sucrose (1:1) were used to collect salivary secretions for approximately 30 min, at which time the mixture was ejected into 0.3 ml MD. Mosquitoes were then placed in individual tubes with MD. All samples were held at −80 °C until tested. Bodies and legs were processed separately as described above and all samples were screened or titrated by duplicate plaque assay on Vero cells.

In vitro growth analyses.

Virus growth analyses were completed basically as described previously (Ciota *et al.*, 2007b) in SUA 5.1 cells derived from *An. gambiae* mosquitoes (Levashina *et al.*, 2001). Briefly, confluent monolayers were infected in duplicate in six-well plates with 100 μl of approximately $10^{7.0}$ p.f.u. (m.o.i. of 1) of virus. Infection was allowed to proceed for 1 h, inoculum was removed, monolayers were washed three times with PBS, and 3 ml Schneider's media (Invitrogen) supplemented with 10% FBS was added. Media was again replaced at 7 h p.i. in order to assure virus measured in supernatant resulted from replication alone. This step was added based on previous experiments in which washing was not sufficient to remove virus bound early to cells (data not shown). This virus appeared to be released later into the supernatant and subsequently distorted measurements of early replication. Virus accumulation was determined on days 1–6 p.i. by removing 100 μl of media and testing by plaque titration on Vero cell culture for WNV and SLEV as described previously (Payne *et al.*, 2006).

Chicken viraemia experiments.

One-day-old chickens were inoculated subcutaneously with 3.5–10 p.f.u. per 100 μl of WNV UNP, WNV CP39, SLEV UNP or SLE CP40. WNV and SLEV experiments were completed separately. Five chickens per virus and two mock-inoculated chickens, i.e. inoculated with animal diluent (PBS/1% FBS) only, were housed separately in adjacent cages and distinguished by colour banding. Chickens were bled from the brachial vein and blood was collected by capillary action in serum separator tubes on days 1–5 p.i. Chickens were monitored for signs of illness and were euthanized using 100 μl Sleepaway (Fort Dodge Laboratories) followed by cervical dislocation upon completion of experiment. Blood was centrifuged at 10000 rcf for 10 min and serum was removed, diluted 1:10 in BA-1 and stored at ̃80 °C until tested. Levels of viraemia were determined by plaque titration on Vero cells as described previously (Payne *et al.*, 2006). All animal use was approved by Wadsworth Center IACUC 03-355.

Molecular cloning.

Generation of molecular clones for the SLEV nucleotide region 738–4202 (M/E/NS1/NS2A) was done as described previously (Jerzak *et al.*, 2005). RNA was

extracted from infected specimens with RNeasy spin columns (Qiagen). RT-PCR reactions were conducted for nucleotide region 738–2465 for SLEV UNP, SLEV CP6, SLEV CP10 and SLEV CP40 using primers designed to amplify the 3′ 738 nt of the SLEV M-coding region and the 5′ 2465 nt of the SLEV E glycoprotein. RT-PCR reactions were conducted for nucleotide region 2466–4202 for SLEV UNP and SLEV CP40, using primers designed to amplify the 3′ 2466 nt of the non-structural (NS) protein 1 coding region and the 5′ 4202 nt of the NS protein 2a (NS2a). RT of 5 μl RNA was performed with Sensiscript RT (Qiagen) at 40 °C for 60 min. RT reactions were followed by heat inactivation at 93 °C for 5 min. The resulting cDNA was used as a template for PCR amplification. SLEV cDNA was then amplified with a ‘high-fidelity’ protocol using *Pfu* Ultra (Stratagene), according to the manufacturer’s specifications. Amplification was carried out for 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 4 min, and one cycle at 72 °C for 10 min. PCR products were visualized on a 1.5% agarose gel and DNA was recovered by using a MiniElute Gel Extraction kit (Qiagen) as specified by the manufacturer. The recovered DNA was ligated into the cloning vector pCR-Script Amp SK(+) and transformed into XL10-Gold Ultracompetent cells (Stratagene) according to the manufacturer’s protocol. The blue–white colour-screening method was used to select transformed colonies. White colonies were screened by direct PCR using primers specific for the desired inserts. Plasmid DNA was purified by using QIAprep Spin Miniprep kit (Qiagen) as specified by the manufacturer. Sequencing was carried out by using five forward and five reverse overlapping primers, T7 forward and the T3 reverse primer for the M and E regions, as well as six forward and six reverse overlapping primers, T7 forward and the T3 reverse primer for the NS1 and NS2a regions. Sequencing was performed at the Wadsworth Center Molecular Genetics Core using ABI 3700 and 3100 automated sequencers (Applied Biosystems). Twenty-one to 24 clones per sample were sequenced.

Data analysis.

Sequences were compiled and edited using the SeqMan module of the DNASTAR software package and a minimum of twofold redundancy throughout each clone was required for sequence data to be considered complete. Twenty-one to 24 clones from each individual sample were aligned using MEGALIGN within DNASTAR. The consensus sequence for each sample was determined and the sequence of each clone was compared to the population consensus. The percentage of nucleotide mutations (total number of mutations divided by total number of bases sequenced) was used as an indicator of genetic diversity. Statistic analyses were performed using both Microsoft Excel 2003 and GraphPad Prism version 4.03.

RESULTS

Cx. pipiens vector competence

Infection, dissemination and transmission rates were determined in *Cx. pipiens* mosquitoes for C6/36-adapted viruses (WNV CP39 and SLEV CP40) as compared to unpassaged viruses [WNV UNP (Table 1) and SLEV UNP (Table 2)]. Bloodmeal titres of WNV UNP and WNV CP39 used in feeding experiments were 7.8 and 7.7 $\log_{10}$ p.f.u. ml^{-1}, respectively. Infection rates overall were 28% for WNV CP39 and 21% for WNV UNP (Table 1). Although per cent infected was higher for WNV CP39, significant differences (Fisher's exact) in infection did not exist overall or on any individual day (Fisher's exact, $P>0.05$). Dissemination and transmission rates overall and on individual days were also not significantly different ($P>0.05$). In addition, body titres for WNV UNP and WNV CP39 were statistically similar on all days p.i. (t-test, $P>0.05$). No consistent significant differences between passaged and unpassaged viruses were identified in two replicates of these experiments (data not shown). Bloodmeal titres of SLEV in feeding studies were 6.8 $\log_{10}$ p.f.u. ml^{-1} for SLEV CP40 and 6.5 $\log_{10}$ p.f.u. ml^{-1} for SLEV UNP. SLEV CP40 did display lower infection rates than SLEV UNP on all days tested (Table 2), but they were not statistically significant on any individual days (Fisher's exact, $P>0.05$). Overall infection rates of SLEV CP40 were significantly lower than SLEV UNP (Fisher's exact, $P=0.018$). Dissemination and transmission rates for SLEV CP40 were also statistically lower than SLEV UNP on day 15 ($P<0.05$). Body titres were lower for SLEV CP40 on all days tested and differences were statistically significant on days 7, 9 and 15 p.i. (t-test, $P<0.05$).

Virus growth in *Cx. pipiens*

Replication of WNV UNP and SLEV UNP was compared with WNV CP39 and SLEV CP40 in *Cx. pipiens* mosquitoes following intrathoracic inoculation of 10–20 times the ID_{50} of individual virus strains in order to determine if virus growth advantages in C6/36 cells translated to changes in virus growth *in vivo* (Fig. 1). The ID_{50} values were similar among virus populations: 1.04 (WNV UNP), 0.95 (WNV CP39), 0.30 (SLEV UNP) and 0.10 p.f.u. (SLEV CP40). In general, SLEV growth, but not WNV, appeared attenuated in *Cx. pipiens* following inoculation of mosquito cell-adapted viruses (Fig. 1). SLEV body titres on days 1 and 2 p.i. were equivalent for both groups, but titres began to diverge on day 3 p.i. (0.4 $\log_{10}$ p.f.u. per mosquito difference). Body titres for SLEV CP40-infected mosquitoes were statistically lower (t-test, $P<0.01$) than SLEV UNP-infected mosquitoes by day 4 p.i. (0.7 $\log_{10}$ p.f.u. per mosquito difference) and this difference increased to 2.0 $\log_{10}$ p.f.u. per mosquito by 7 days p.i. (peak titre for SLEV UNP). Following 7 days, virus titres for SLEV

UNP-infected mosquitoes gradually declined, while titres for SLEV CP40-infected mosquitoes continued to gradually increase. Nonetheless, differences remained statistically significant throughout the experiment. Virus growth in WNV-infected mosquitoes was substantially different from SLEV (Fig. 1). Significantly lower body titres for WNV CP39-infected mosquitoes were measured on day 1 p.i. (*t*-test, $P<0.001$). This difference was likely greater than the reported difference because virus was undetectable on day 1 p.i. in all mosquitoes inoculated with WNV CP39, and values were therefore set to the assay limit of detection (10 p.f.u. per mosquito). By day 2 p.i., WNV CP39 virus growth rebounded and reached statistically equivalent titres to that of WNV UNP. Statistical equivalence remained through to day 21 p.i.

Chicken viraemia

One-day-old chickens were inoculated subcutaneously with 4–15 p.f.u. per 100 μl of the following virus populations: SLEV UNP, SLEV CP40, WNV UNP and WNV CP39.

All chickens inoculated in each experiment became viraemic. SLEV serum titres on days 1–4 p.i. displayed a slower growth rate and lower peak viraemia following passage in mosquito cell culture (Fig. 2). Statistically significant differences were measured on days 2 and 4 p.i. (*t*-test, $P<0.05$), although titres were also lower on days 1 and 3 p.i. One of five (SLEV CP40) and four of five (SLEV UNP) chickens displayed viraemia titres that were below the assay level of detection (50 p.f.u. ml^{-1} on day 1 p.i. and were therefore set to this level. For this reason, the actual difference in viraemia at this time point may be greater.

No significant differences between serum titres for WNV CP39- and WNV UNP-infected chickens were measured on any day p.i (Fig. 2). Although values were generally lower on day 1 p.i. for WNV CP39, in a trend similar to virus growth in mosquitoes, rebounded to equivalent levels by day 2 p.i.

In vitro viral growth analyses

Growth patterns of WNV CP39 and SLEV CP40, along with unpassaged controls, were determined in SUA 5.1 cells derived from *An. gambia* mosquitoes in order to determine if replicative advantages described previously in C6/36 cells (Ciota *et al.*, 2007a) translated to changes in replication in other mosquito cell lines (Fig. 3). Both initial growth rate and peak titre were significantly greater for WNV CP39 relative to WNV UNP (*t*-test, $P<0.05$). Differences of greater than 2 $\log_{10}$ p.f.u. ml^{-1} were measured at day 1 p.i. Although this difference decreased in subsequent days, peak titre for WNV CP39 (day 5 p.i.) remains approximately 1 $\log_{10}$ p.f.u. ml^{-1} greater than WNV UNP and statistical significance exists on all days (*t*-test, $P<.05$). Results for C6/36-adapted SLEV are strikingly different (Fig. 3). Growth of SLEV CP40 is significantly attenuated in SUA 5.1 cells. Although only a small

difference in mean titre was measured on day 1 p.i. (0.5 $\log_{10}$ p.f.u.; *t*-test, $P=0.045$), by day 2 p.i. SLEV mean titre is substantially higher (1.5 $\log_{10}$ p.f.u.; *t*-test, $P=0.0014$). No significant growth occurred for SLEV CP40 beyond day 2 p.i., whereas growth of SLEV UNP continued throughout the experiment. Statistically significant differences were measured on all days p.i. (*t*-test, $P<0.05$).

Genetic diversity of SLEV populations following cell culture passage

Genetic diversity of the following SLEV strains was determined by clonal analyses: SLEV UNP, C6/36 passage 6 (SLEV CP6), C6/36 passage 10 (SLEV CP10), C6/36 passage 40 (SLEV CP40). Two regions including nt 738–4202 composed of the M, E, NS1 and NS2A genes were analysed for SLEV UNP and SLEV CP40, while only nt 738–2465 (M/E) were analysed for SLEV CP6 and SLEV CP10. SLEV UNP was found to be a fairly homogeneous population with nucleotide diversity of 0.058% for all regions analysed (Table 3). This amounted to a total of 10 base differences relative to consensus found in four of 23 sequences analysed. Genetic homogeneity was anticipated for this population since this virus strain was itself a biological clone. To measure the level of diversity resulting from sequential C6/36 passage and adaptation, genetic diversity of SLEV CP40 was determined. Results indicated an increase in homogeneity from unpassaged SLEV, with SLEV CP40 being a highly homogeneous population possessing just two total base differences identified in approximately 80000 bases sequenced.

Analyses of SLEV CP6 and SLEV CP10 were completed to determine if there was an intermediate increase in genetic diversity during passage. Although only portions of the M- and E-coding regions were analysed for these populations, previous results indicated that these regions would likely provide an accurate representation of genome diversity or, if biased at all, would bias towards diversity. Results again indicated high levels of homogeneity for both populations on nucleotide and amino acid levels, with a slight decrease in diversity from passage 6 to 10 (Table 3). The overall trend, in fact, indicated that the small number of variants that initially existed in the unpassaged populations were gradually purged with subsequent mosquito cell passage.

DISCUSSION

Cell culture systems are widely used to study arbovirus adaptation and fitness (Domingo & Holland, 1997; Cooper & Scott, 2001; Holland *et al.*, 1991; Novella *et al.*, 1999a, b; Weaver *et al.*, 1999; Ciota *et al.*, 2007a; Chen *et al.*, 2003; Zarate & Novella, 2004).

Although there are numerous previous studies demonstrating that *in vivo* attenuation of virus replication may be attained by cell culture passage (Miller & Mitchell, 1986; Barrett *et al.*, 1990; Halstead *et al.*, 1984; Hearn, *et al.*, 1966; Eckels *et al.*, 1984; Goto *et al.*, 2003), whether arbovirus adaptations to cells derived from a relevant insect host translate to phenotypic changes *in vivo* in both mosquito and avian hosts has not been directly evaluated. Here, we measured both vector competence and virus growth of mosquito cell-adapted WNV and SLEV in *Cx. pipiens* mosquitoes. Our results indicate for both viruses that adaptations to mosquito cells did not translate to *in vivo* adaptations to *Cx. pipiens* mosquitoes. The lack of adaptation measured *in vivo* can likely be attributed to the differences in the complexity of a mosquito relative to that of a limited cell type *in vitro.* Specifically, viral fitness within the mosquito depends on both the ability to infect and replicate in numerous tissue types, and to successfully cross both midgut and salivary gland barriers (Kramer *et al.*, 1981; Woodring *et al.*, 1996). Although this result was expected, it underscores the importance of *in vivo* experimentation in characterizing arbovirus adaptation.

The differences between SLEV and WNV following *in vitro* adaptation are intriguing. These two flaviviruses are very closely related genetically, structurally and ecologically (Monath & Heinz, 1996); thus, the expectation was that they would react very similarly to identical cell culture passage. Our previous studies indicated that although both viruses displayed significant cell-specific adaptation following passage, the rate and magnitude of SLEV adaptation was greater than that of WNV (Ciota *et al.*, 2007a). Given that viruses were passaged in an *Ae. albopictus* cell line (C6/36), the specificity of the adaptations was assessed here with *in vitro* growth experiments in SUA 5.1 cells (Levashina *et al.*, 2001), derived from the genetically distant *An. gambia* species (Rao & Rai, 1990). Results indicated that growth advantages in C6/36 could be generalized to these cells for WNV but not SLEV (Fig. 3). This suggests that the specificity of adaptation is greater for SLEV. Results presented here also show that cell culture adaptation of SLEV resulted in attenuated growth *in vivo*, while for WNV, adaptation generally did not (Table 1, Fig. 1). Specifically, no differences in infection rate, dissemination rate or body titre were measured for WNV CP39 as compared to WNV UNP in *Cx. pipiens* at any day following feeding on infectious bloodmeals (Table 1). SLEV CP40, on the other hand, displayed significantly lower overall infection and dissemination rates, and body titres on all days tested post-feeding. Previous studies with dengue virus demonstrated that passage in C6/36 cell culture can significantly affect infectivity and virus dissemination in *Ae. aegypti* mosquitoes (Schoepp *et al.*, 1990; Bosio *et al.*, 2000). Virus growth analysis of SLEV CP40 following inoculation showed similar results, with statistically lower titres measured

at days 4–21 p.i (Fig. 1). WNV CP39, although replicating to statistically lower titres on day 1 p.i., subsequently displayed accelerated growth and titres that were statistically equivalent to WNV UNP. This early difference in replication could be explained by the fact that the dominant *in vitro*-adapted genotypes may be somewhat attenuated *in vivo*, and the population's ability to then rebound to normal levels of replication could then be attributed to the previously reported existence of multiple genotypic variants within the mosquito cell passaged WNV population (Ciota *et al.*, 2007b) . Studies with FMDV showed that variants initially existing in low numbers in the population could quickly become the master sequence upon entry into a different environment (Martinez *et al.*, 1991). This could also explain the WNV viraemia in chickens, in which no cost in replicative ability was measured for WNV CP39 (Fig. 2). The benefit of genetic diversity in a population's ability to quickly adapt to new and changing environments is clear. This explanation would predict that a population such as SLEV CP40, in which attenuated growth was measured throughout experiments in both mosquito (Fig. 1) and chicken (Fig. 2) environments, does not possess the adaptive benefit of a large mutant spectrum. Our results were consistent with this explanation. The levels of nucleotide diversity for SLEV UNP, SLEV CP6, SLEV CP10 and SLEV CP40 were not significantly different from each other and, in fact, tended toward increased homogenity with further passage (Table 3). The level of homogeneity within SLEV populations was confirmed in a separate lineage of C6/36 passaged virus. This lack of heterogenity is substantially different from the previously reported results for C6/36 passaged WNV, in which diversity increased in a linear fashion with passage and adaptation, and ultimately resulted in the highly heterogeneous WNV CP39 (Ciota *et al.*, 2007b) used in this study. The study by Ciota *et al.* (2007b), showed that 87% of clones analysed for WNV CP39 were different from the consensus sequence, whereas just 3% of SLEV CP40 clones measured here had mutations. This again suggests the selective pressures applied by this particular *in vitro* environment are stronger and perhaps more specific for SLEV. Given the mutation rate of approximately 1 mutation in 10^4 bp replicated that presumably exists with RNA viruses (Drake & Holland, 1999), the level of selection must have been high to maintain and, perhaps decrease homogeneity with mosquito cell passage. This is consistent with the higher rate and magnitude of adaptation measured in SLEV relative to WNV, and the fact that more consensus changes were identified in SLEV (Ciota *et al.*, 2007a). These differences are also consistent with the recent epidemiological patterns of virus activity observed with SLEV and WNV in North America. WNV has been highly successful since its 1999 introduction to New York, with continued expansion in both geographical and host range (Davis *et al.*, 2005; http://www.cdc.gov/ncidod/dvbid/arbor). SLEV, on the other hand, continues to be subjected to periodic outbreaks, which generally remain isolated with relatively low levels

of activity (Reisen, 2003; Monath & Heinz, 1996; http://www.cdc.gov/ncidod/dvbid/arbor). Whether or not WNV activity will ultimately mimic that of SLEV in North America remains to be seen, but these current differences could certainly be contributed to, both by differences in the viruses' ability to produce and maintain adaptable populations and variable levels of specificity in virus adaptation. The frequently lower viraemia titres in avian hosts for SLEV as compared with WNV (Reisen *et al.*, 2005; McLean *et al.*, 2001; Fig. 2) is clearly important in driving the intensity of transmission. Differences in adaptability and plasticity presented here could partially explain these viraemia differences. Cell culture studies suggest that the more persistent phase of the transmission cycle (i.e. the vector) dominates evolutionary pressures for VSV (Zarate & Novella, 2004). Our studies demonstrate that SLEV and WNV are equally well adapted to replication in the mosquito (Fig. 1), therefore adaptation to the mosquito may result in a replicative cost in the avian environment for SLEV but not WNV. Substantial genetic diversity has been identified previously in natural isolates of WNV (Jerzak *et al.*, 2007); similar studies with SLEV together with analyses of *in vivo*-adapted populations, will help us to clarify the true importance of these factors in the epidemiology of these and other potentially important arboviruses.

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REFERENCES

Arias, A., Ruiz-Jarabo, C. M., Escarmis, C. & Domingo, E. (2004). Fitness increase of memory genomes in a viral quasispecies. *J Mol Biol* **339**, 405–412. Medline

Austin, R. J., Whiting, T. L., Anderson, R. A. & Drebot, M. A. (2004). An outbreak of West Nile virus-associated disease in domestic geese (*Anser anser domesticus*) upon initial introduction to a geographic region, with evidence of bird to bird transmission. *Can Vet J* **45**, 117–123. Medline

Barrett, A. D., Monath, T. P., Cropp, C. B., Adkins, J. A., Ledger, T. N., Gould, E. A., Schlesinger, J. J., Kinney, R. M. & Trent, D. W. (1990). Attenuation of wild-type yellow fever virus by passage in HeLa cells. *J Gen Virol* **71**, 2301–2306. Medline

Bosio, C. F., Fulton, R. E., Salasek, M. L., Beaty, B. J. & Black, W. C. (2000). Quantitative trait loci that control vector competence for dengue-2 virus in the mosquito *Aedes aegypti. Genetics* **156**, 687–698. Medline

Briones, C., de Vicente, A., Molina-Paris, C. & Domingo, E. (2006). Minority memory genomes can influence the evolution of HIV-1 quasispecies *in vivo*. *Gene* **384**, 129–138. Medline

Chandler, L. J., Parsons, R. & Randle, Y. (2001). Multiple genotypes of St Louis encephalitis virus (Flaviviridae: *Flavivirus*) circulate in Harris County, Texas. *Am J Trop Med Hyg* **64**, 12–19. Medline

Chen, W. J., Wu, H. R. & Chiou, S. S. (2003). E/NS1 modifications of dengue 2 virus after serial passages in mammalian and/or mosquito cells. *Intervirology* **46**, 289–295. Medline

Ciota, A. T., Lovelace, A. O., Ngo, K. A., Le, A., Maffei, J. G., Franke, M. A., Payne, A. F., Jones, S. A., Kauffman, E. B. & other authors (2007a). Cell-specific adaptation of two flaviviruses following serial passage in mosquito cell culture. *Virology* **357**, 165–174. Medline

Ciota, A. T., Ngo, K. A., Lovelace, A. O., Payne, A. F., Zhou, Y., Shi, P.-Y. & Kramer, L. D. (2007b). Role of the mutant spectrum in adaptation and replication of *West Nile virus*. *J Gen Virol* **88**, 865–874. Medline

Cooper, L. A. & Scott, T. W. (2001). Differential evolution of eastern equine encephalitis virus populations in response to host cell type. *Genetics* **157**, 1403–1412. Medline

Cruz, L., Cardenas, V. M., Abarca, M., Rodriguez, T., Reyna, R. F., Serpas, M. V., Fontaine, R. E., Beasley, D. W. C., Travassos da Rosa, A. P. A. & other authors

(2005). Short report: serological evidence of West Nile virus activity in El Salvador. *Am J Trop Med Hyg* **72**, 612–615. Medline

Davis, C. T., Ebel, G. D., Lanciotti, R. S., Brault, A. C., Guzman, H., Siirin, M., Lambert, A., Parsons, R. E., Beasley, D. W. & other authors (2005). Phylogenetic analysis of North American West Nile virus isolates, 2001–2004: evidence for the emergence of a dominant genotype. *Virology* **342**, 252–265. Medline

Day, J. F. & Stark, L. M. (2000). Frequency of Saint Louis encephalitis virus in humans from Florida, USA: 1990–1999. *J Med Entomol* **37**, 626–633. Medline

Domingo, E. & Holland, J. J. (1997). RNA virus mutations and fitness for survival. *Annu Rev Microbiol* **51**, 151–178. Medline

Drake, J. W. & Holland, J. J. (1999). Mutation rates among RNA viruses. *Proc Natl Acad Sci U S A* **96**, 13910–13913. Medline

Dupuis, A. P., Marra, P. P. & Kramer, L. D. (2003). Serologic evidence of West Nile virus transmission, Jamaica, West Indies. *Emerg Infect Dis* **9**, 860–863. Medline

Dupuis, A. P., Marra, P. P., Reitsma, R., Jones, M. J., Louie, K. L. & Kramer, L. D. (2005). Serologic evidence for West Nile virus transmission in Puerto Rico and Cuba. *Am J Trop Med Hyg* **73**, 474–476. Medline

Ebel, G. D., Rochlin, I., Longacker, J. & Kramer, L. D. (2005). *Culex restuans* (Diptera:culicidae) relative abundance and vector competence for West Nile virus. *J Med Entomol* **42**, 838–843. Medline

Eckels, K. H., Scott, R. M., Bancroft, W. H., Brown, J., Dubois, D. R., Summers, P. L., Russell, P. K. & Halstead, S. B. (1984). Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. V. Human response to immunization with a candidate vaccine prepared in fetal rhesus lung cells. *Am J Trop Med Hyg* **33**, 684–689. Medline

Elizondo-Quiroga, D. (2005). West nile virus isolation in human and mosquitoes, Mexico. *Emerg Infect Dis* **11**, 1449–1452. Medline

Goto, A., Hayasaka, D., Yoshii, K., Mizutani, T., Kariwa, H. & Takashima, I. (2003). A BHK-21 cell culture-adapted tick-borne encephalitis virus mutant is attenuated for neuroinvasiveness. *Vaccine* **21**, 4043–4051. Medline

Granwehr, B. P., Lillibridge, K. M., Higgs, S., Mason, P. W., Aronson, J. F., Campbell, G. A. & Barrett, A. D. T. (2004). West Nile virus: where are we now? *Lancet Infect Dis* **4**, 547–556. Medline

Halstead, S. B., Diwan, A. R., Marchette, N. J., Palumbo, N. E. & Srisukonth, L. (1984). Selection of attenuated dengue 4 viruses by serial passage in primary kidney cells. I. Attributes of uncloned virus at different passage levels. *Am J Trop Med Hyg* **33**, 654–665. Medline

Hearn, H. J., Jr, Chappell, W. A., Demchak, P. & Kominik, J. W. (1966). Attenuation of aerosolized yellow fever virus after passage in cell culture. *Bacteriol Rev* **30**, 615–623. Medline

Higgs, S., Snow, K. & Gould, E. A. (2004). The potential for West Nile virus to establish outside of its natural range: a consideration of potential mosquito vectors in the United Kingdom. *Trans R Soc Trop Med Hyg* **98**, 82–87. Medline

Holland, J. J., De La Torre, J. C., Clarke, D. K. & Duarte, E. (1991). Quantitation of relative fitness and great adaptability of clonal populations of RNA viruses. *J Virol* **65**, 2960–2967. Medline

Jerzak, G., Bernard, K. A., Kramer, L. D. & Ebel, G. D. (2005). Genetic variation in *West Nile virus* from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. *J Gen Virol* **86**, 2175–2183. Medline

Jerzak, G. V., Bernard, K., Kramer, L. D., Shi, P. Y. & Ebel, G. D. (2007). The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. *Virology* **360**, 469–476. Medline

Kramer, L. D. & Bernard, K. A. (2001). West Nile virus infection in birds and mammals. *Ann N Y Acad Sci* **951**, 84–93. Medline

Kramer, L. D., Hardy, J. L., Presser, S. B. & Houk, E. J. (1981). Dissemination barriers for western equine encephalomyelitis virus in *Culex tarsalis* infected after ingestion of low viral doses. *Am J Trop Med Hyg* **30**, 190–197. Medline

Lanciotti, R. S., Roehrig, J. T., Deubel, V., Smith, J., Parker, M., Steele, K., Crise, B., Volpe, K. E., Crabtree, M. B. & other authors (1999). Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**, 2333–2337. Medline

Levashina, E. A., Moita, L. F., Blandin, S., Vriend, G., Lagueux, M. & Kafatos, F. C. (2001). Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell* **104**, 709–718. Medline

Marra, P. P., Griffing, S. M. & McLean, R. G. (2003). West Nile virus and wildlife health. *Emerg Infect Dis* **9**, 898–899. Medline

Martinez, M. A., Carrillo, C., Gonzalez-Candelas, F., Moya, A., Domingo, E. & Sobrino, F. (1991). Fitness alteration of foot-and-mouth disease virus mutants: measurement of adaptability of viral quasispecies. *J Virol* **65**, 3954–3957. Medline

McLean, R. G., Ubico, S. R., Docherty, D. E., Hansen, W. R., Sileo, L. & McNamara, T. S. (2001). West Nile virus transmission and ecology in birds. *Ann N Y Acad Sci* **951**, 54–57. Medline

Miller, B. R. & Mitchell, C. J. (1986). Passage of yellow fever virus: its effect on infection and transmission rates in *Aedes aegypti. Am J Trop Med Hyg* **35**, 1302–1309. Medline

Monath, T. P. & Heinz, F. X. (1996). Flaviviruses. In *Fields Virology*, 3rd edn, pp. 961–1034. Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams and Wilkins.

Morales, M. A., Barrandeguy, M., Fabbri, C., Garcia, J. B., Vissani, A., Trono, K., Gutierrez, G., Pigretti, S., Menchaca, H. & other authors (2006). West Nile virus isolation from equines in Argentina, 2006. *Emerg Infect Dis* **12**, 1559–1561. Medline

Novella, I. S., Hershey, C. L., Escarmis, C., Domingo, E. & Holland, J. J. (1999a). Lack of evolutionary stasis during alternating replication of an arbovirus in insect and mammalian cells. *J Mol Biol* **287**, 459–465. Medline

Novella, I. S., Quer, J., Domingo, E. & Holland, J. J. (1999b). Exponential fitness gains of RNA virus populations are limited by bottleneck effects. *J Virol* **73**, 1668–1671. Medline

Payne, A. F., Binduga-Gajewska, I., Kauffman, E. B. & Kramer, L. D. (2006). Quantitation of flaviviruses by fluorescent focus assay. *J Virol Methods* **134**, 183–187. Medline

Rao, P. N. & Rai, K. S. (1990). Genome evolution in the mosquitoes and other closely related members of superfamily Culicoidea. *Hereditas* **113**, 139–144. Medline

Reisen, W. K. (2003). Epidemiology of St Louis encephalitis virus. *Adv Virus Res* **61**, 139–183. Medline

Reisen, W. K., Fang, Y. & Martinez, V. M. (2005). Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St Louis encephalitis virus transmission. *J Med Entomol* **42**, 367–375. Medline

Rosen, L. & Gubler, D. (1974). The use of mosquitoes to detect and propagate dengue viruses. *Am J Trop Med Hyg* **23**, 1153–1160. Medline

Ruiz-Jarabo, C. M., Arias, A., Baranowski, E., Escarmis, C. & Domingo, E. (2000). Memory in viral quasispecies. *J Virol* **74**, 3543–3547. Medline

Schneider, W. L. & Roossinck, M. J. (2000). Evolutionarily related Sindbis-like plant viruses maintain different levels of population diversity in a common host. *J Virol* **74**, 3130–3134. Medline

Schoepp, R. J., Beaty, B. J. & Eckels, K. H. (1990). Dengue 3 virus infection of Aedes albopictus and Aedes aegypti: comparison of parent and progeny candidate vaccine viruses. *Am J Trop Med Hyg* **42**, 89–96. Medline

Weaver, S. C., Brault, A. C., Kang, W. & Holland, J. J. (1999). Genetic and fitness changes accompanying adaptation of an arbovirus to vertebrate and invertebrate cells. *J Virol* **73**, 4316–4326. Medline

Woodring, J. L., Higgs, S. & Beaty, B. J. (1996). Natural cycles of vector-borne pathogens. In *The Biology of Disease Vectors*, pp. 51–72. Edited by B. J. Beaty & W. C. Marquardt. Niwot: University Press of Colorado.

Zarate, S. & Novella, I. S. (2004). Vesicular stomatitis virus evolution during alternation between persistent infection in insect cells and acute infection in mammalian cells is dominated by the persistence phase. *J Virol* **78**, 12236–12242. Medline

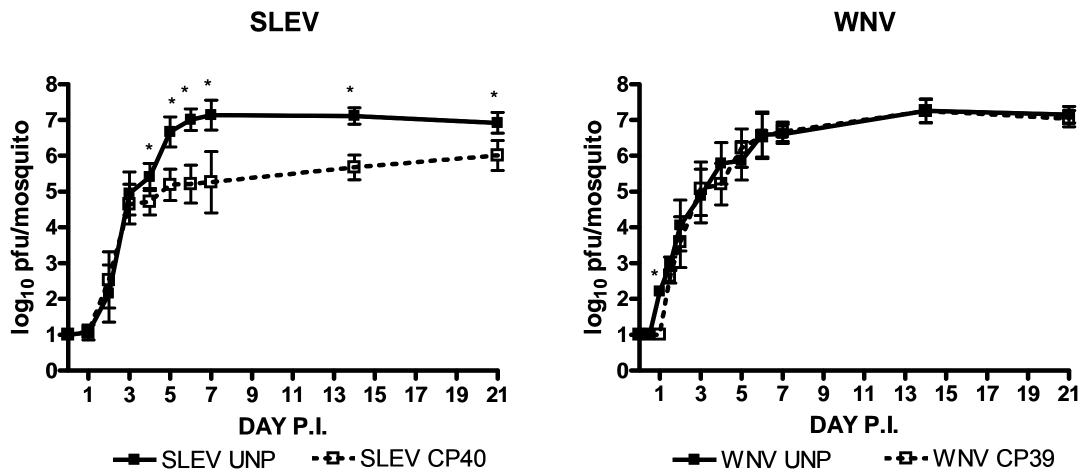

Fig. 1. SLEV and WNV growth in *Cx. pipiens* mosquitoes following intrathoracic inoculation of unpassaged (UNP) or C6/36 passaged (CP) viruses. Results are presented as mean titres of 8–10 mosquitoes per time point±standard deviation (SD). Statistically significant differences (*t*-test, $P<0.05$) are denoted with *.

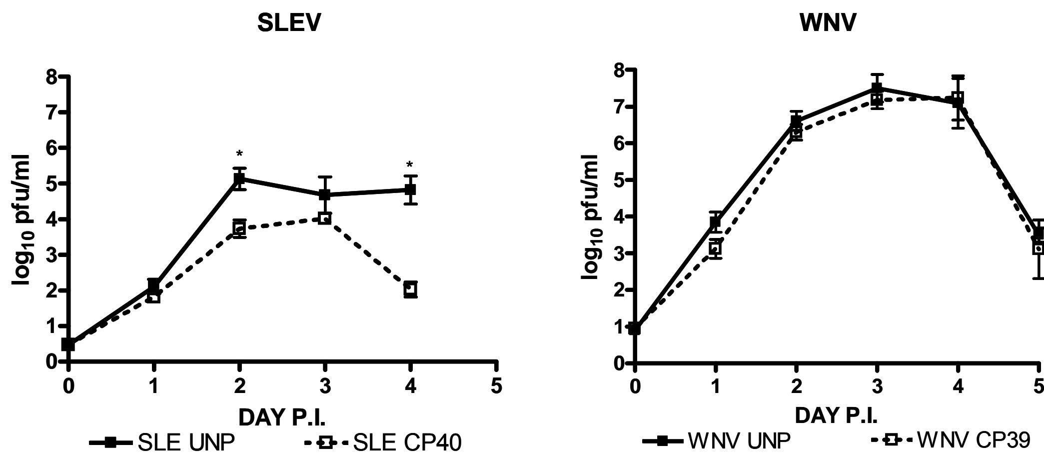

Fig. 2. SLEV and WNV viraemia levels in chicken serum following subcutaneous inoculation of unpassaged (UNP) or C6/36 passaged (CP) viruses. Results are presented as mean titres of 5–6 chickens per time point±SD. Statistically significant differences (*t*-test, $P<0.05$) are denoted with *.

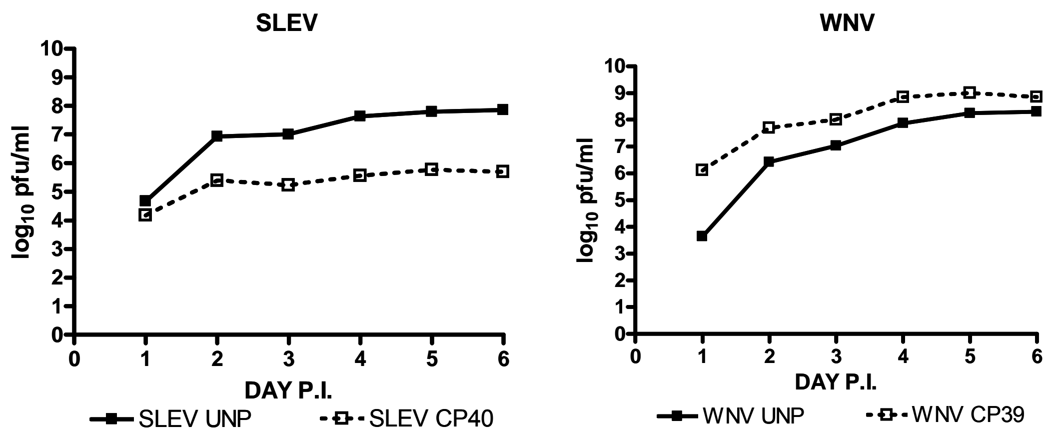

Fig. 3. SLEV and WNV growth in SUA 5.1 cell culture (*An. gambiae*). Results are means of duplicate assays±SD. m.o.i. for all growth curves is 1 p.f.u. per cell. Statistically significant differences (*t*-test, $P<0.05$) were measured on all days for both viruses.

Table 1. Vector competence of *Cx. pipiens* mosquitoes on various days following feeding on infectious bloodmeals containing WNV UNP or WNV CP39

WNV strain	Day	Infected (%; *n*)	WNV titres*	Infected disseminating (%)	Infected transmitting (%)
UNP	5	25 (10)	2.9±1.0	0	–
CP39	5	25 (10)	2.7±1.1	0	–
P value	–	*1.00*	*0.586*	–	–
UNP	7	25(10)	3.3±0.86	10	–
CP39	7	32 (16)	3.6±0.60	0	–
P value	*0.254*	*0.652*	*0.385*	–	–
UNP	9	23 (9)	4.0±1.7	13	22
CP39	9	32 (16)	3.7±0.48	22	6
P value	–	0.*353*	*0.847*	*0.602*	*0.549*
UNP	15	25 (6)	3.2±0.66	17	17
CP39	15	29 (10)	3.5±1.5	20	10
P value	–	*0.773*	*0.875*	*1.00*	*1.00*
UNP	All	21 (35)	NA	NA	NA
CP39	All	28 (52)	NA	NA	NA
P value	–	*0.140*	NA	NA	NA

*Mean titres of infected mosquitoes (p.f.u. per mosquito)±SD.

NA, Not applicable.

Table 2. Vector competence of *Cx. pipiens* mosquitoes on various days following feeding on infectious bloodmeals containing SLEV UNP or SLEV CP40

SLEV strain	Day	Infected (%; *n*)	SLEV titres*	Infected disseminating (%)	Infected transmitting (%)
UNP	5	24 (12)	3.8±0.75	–	0
CP40	5	8 (4)	3.2±0.16	–	0
P value	–	*0.054*	*0.124*	–	–
UNP	7	26 (13)	4.1±0.67	0	–
CP40	7	20 (10)	3.2±0.19	0	–
P value	–	*0.635*	*0.011*	–	–
UNP	9	30 (15)	4.5±0.72	20 (3)	7 (1)
CP40	9	16 (8)	3.6±0.25	0	0
P value	–	*0.153*	*0.012*	*0.526*	*1.00*
UNP	15	22 (11)	5.2±0.48	55 (6)	45 (5)
CP40	15	18 (9)	3.4±0.36	0	0
P value	–	*0.803*	*<0.001*	*0.014*	*0.038*
UNP	All	26 (51)	NA	NA	NA
CP40	All	16 (31)	NA	NA	NA
P value	–	*0.018*	NA	NA	NA

*Mean titres of infected mosquitoes (p.f.u. per mosquito)±SD.

NA, Not applicable.

Table 3. Per cent nucleotide diversity in various coding regions of SLEV populations

SLEV population (# clones)	All regions	M (738–962)	ENV (963–2465)	NS1 (2466–3707)	NS2A (3708–4202)
SLEV UNP (23)	0.012%	0.058%	0.006%	0.007%	0.026%
SLEV C6/36 passage 6 (21)	0.010%	0.021%	0.010%	ND	ND
SLEV C6/36 passage 10 (24)	0.004%	0%	0.006%	ND	ND
SLEV C6/36 passage 40 (24)	0.002%	0%	0%	0.003%	0.008%

ND, Not determined.