Consequences of in vitro host shift for St. Louis encephalitis virus

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Understanding the potential for host range shifts and expansions of RNA viruses is critical to predicting the evolutionary and epidemiological paths of these pathogens. As arthropod-borne viruses (arboviruses) experience frequent spillover from their amplification cycles and are generalists by nature, they are likely to experience a relatively high frequency of success in a range of host environments. Despite this, the potential for host expansion, the genetic correlates of adaptation to novel environments and the costs of such adaptations in originally competent hosts are still not characterized fully for arboviruses. In the studies presented here, we utilized experimental evolution of St. Louis encephalitis virus (SLEV; family Flaviviridae, genus Flavivirus) in vitro in the Dermacentor andersoni line of tick cells to model adaptation to a novel invertebrate host. Our results demonstrated that levels of adaptation and costs in alternate hosts are highly variable among lineages, but also that significant fitness increases in tick cells are achievable with only modest change in consensus genetic sequence. In addition, although accumulation of diversity may at times buffer against phenotypic costs within the SLEV swarm, an increased proportion of variants with an impaired capacity to infect and spread on vertebrate cell culture accumulated with tick cell passage. Isolation and characterization of a subset of these variants implicates the NS3 gene as an important host range determinant for SLEV.

INTRODUCTION

Rapid, error-prone replication provides RNA viruses with abundant genetic diversity and, consequently, evolutionary potential. Arthropod-borne viruses (arboviruses) are unique among RNA viruses in their capacity to successfully propagate in, and be transmitted by, divergent vertebrate and invertebrate hosts. The requirement for host cycling could result in a predisposition for plasticity, permitting host range expansion without a cost. Here, we utilized experimental evolution of SLEV in vitro in Dermacentor andersoni tick cells to model adaptation to a novel invertebrate host. Our results demonstrated that levels of adaptation and costs in alternate hosts are highly variable among lineages, but also that significant fitness increases in tick cells are achievable with only modest change in consensus genetic sequence. In addition, although accumulation of diversity may at times buffer against phenotypic costs within the SLEV swarm, an increased proportion of variants with an impaired capacity to infect and spread on vertebrate cell culture accumulated with tick cell passage. Isolation and characterization of a subset of these variants implicates the NS3 gene as an important host range determinant for SLEV.

RESULTS

Growth kinetics and relative fitness

Despite lineage variability, a general trend of increased production of SLEV RNA, consistent with adaptation, was
measured throughout the first nine passages in DAE cells, followed by equilibration of RNA production in which the SLEV RNA level (genomes ml$^{-1}$) fluctuated modestly around a mean of $\sim 6.5 \log_{10} \text{ml}^{-1}$ (Fig. 1). On average, lower titres were measured in lineage C relative to lineages A and B. Following 17 (B) or 19 (A and C) passages, growth kinetics were evaluated in DAE cell culture and compared to unpassaged SLEV WT. Despite evidence of increases in RNA production (Fig. 1), peak production of infectious particles on DAE cells as measured by plaque titration on Vero cell culture was not significantly improved relative to WT, with the exception of strain 17B, for which viral titre was significantly higher than WT at both 96 and 120 h post-infection (p.i.) ($t$-test, $P<0.05$; Fig. 2).

Competition assays were performed in order to more accurately measure differences in replicative fitness in tick cells, and to assess the extent of fitness costs in mosquito and mammalian cell lines resulting from tick cell passage. These assays were only performed after passages 10 and 15, since no evidence of further adaptation was indicated by output RNA levels in later passages (Fig. 1). Unlike kinetics data, evidence for increased fitness in tick cell culture was measured by competition assays for all lineages following both 10 and 15 passages (one-sample $t$-test; $P<0.05$; Fig. 3). Despite this consistent trend, fitness alterations in other host cell lines were variable. For passage 10 strains, the fitness advantage in DAE does not correspond to decreased fitness in C6/36 or Vero cell culture and, in fact, significant increases relative to WT were measured in these lines for strain 10C. Passage 15 strains, however, did accrue fitness losses, although inconsistent, in divergent hosts in vitro. A significant decrease in relative fitness was measured in Vero cell culture for lineages A and C, and a decrease in relative fitness in C6/36 was measured for lineage B ($t$-test, $P<0.05$). In agreement with the significant increase in titre measured with lineage B growth in DAE cells (Fig. 2), relative fitness of lineage B in DAE was significantly higher than all other passaged strains ($t$-test, $P<0.001$; Fig. 3).

Efforts to amplify SLEV in an alternate tick cell line derived from *Ixodes scapularis* (ISE6 cells) prior to DAE passaging demonstrated that these cells are not permissive to the
SLEV WT strain, with no increase in titre relative to input measured in cell supernatant at 6 days p.i. (Fig. 4). In order to determine whether passage of SLEV in DAE resulted in an increased capacity for infection and replication in ISE6, SLEV titres of P19A, P17B and P19C were also quantified in ISE6 cell supernatant at 6 days p.i. Results indicated a modest but significant increase in viral titre relative to input for lineages A and B, consistent with the capacity of ISE6 to sustain a low level of replication of DAE passaged strains (t-test, \( P<0.05 \); Fig. 4).

**Virus infectivity and spread**

An increasing frequency of smaller plaques was observed with Vero cell titration during SLEV passaging on DAE cells. To test whether differences in plaque size were temperature dependent, plaque assays were performed with passaged strains at both 28 and 37 °C. Results of these assays demonstrated that similar differences in plaque size existed at both temperatures (data not shown). Measurement of foci following fluorescent focus assay was used for accurate quantification of these apparent phenotypic alterations. Results confirmed that the capacity for SLEV cell-to-cell spread in mammalian culture was on average delayed or restricted as a result of passage on tick cells (Fig. 5). Specifically, mean focus size on Vero cell culture was smaller relative to WT for lineage A and B strains following 10 passages, and for all three lineages at the completion of the passage series (Mann–Whitney, \( P<0.05 \)). In addition, combined data demonstrated a decrease in foci size with passage (Fig. 5; linear regression analysis, \( R^2=0.57, P=0.0182 \)).

In order to test the hypothesis that SLEV passage in tick cell culture resulted in decreased infectivity in mammalian cell culture, we compared ratios of Vero infectious units (p.f.u.) to viral RNA as quantified by quantitative reverse transcriptase (qRT)-PCR for passages 1, 10 and 17–19. Results confirmed a decrease in mean infectiousness with passage (Fig. 6; one-way ANOVA, \( P=0.0084, F=8.018, R^2=0.62 \)). These data, combined with the plaque/foci size reduction observed with passage, suggest some variants that retained the capacity to infect and replicate in tick cell culture may have lost the capacity to infect and/or spread in mammalian cell culture. In an attempt to isolate clonal populations of these host-restricted variants, limiting dilutions containing ~100 RNA genomes of strain 17A (the lineage with the most significant fitness loss in Vero cell culture; Fig. 3) were amplified individually on DAE cells and Vero p.f.u./RNA ratios were quantified. Results indicated significant intrapopulation phenotypic
variability, with titres ranging from below the limit of detection (5 p.f.u. ml\(^{-1}\)) to 7.66 log\(_{10}\) p.f.u. ml\(^{-1}\) and RNA levels ranging from 4.95 to 8.82 log\(_{10}\) genomes ml\(^{-1}\) (Table 1). Vero p.f.u./RNA ratios also varied substantially and two strains, 17A-9 and 17A-18, were chosen for further characterization due to their impaired capacity to generate plaques on mammalian cell culture. Strain 17A-9 produced no visible plaques by standard plaque assay on Vero cell culture and also grew to relatively lower levels on DAE cells (as indicated by RNA levels). To confirm this impaired infectivity and further compare with infectivity on tick cell culture, fluorescent focus assays were completed on both Vero and DAE cell culture using both SLEV WT and 17A-9. Results demonstrated robust focus development for SLEV WT on Vero cells with more limited foci on DAE cells, and confirmed highly impaired infectivity for 17A-9 on Vero cells with enhanced signal for this strain on DAE cells (Fig. 7).

**Full-genome sequencing**

In order to identify genetic correlates of distinct phenotypes, four SLEV strains, including 15B, 17A, 17A-9 and 17A-18, were chosen for full-genome sequencing and compared to SLEV WT (GenBank accession number DQ525916). SLEV 15B, the strain demonstrating the most significant gains in relative fitness on tick cells (Fig. 3), acquired three consensus substitutions, two of which were non-synonymous (Table 2). The substitutions included a silent change in the NS5 gene (viral polymerase), a serine to isoleucine substitution at aa 85 of the envelope and a valine to isoleucine substitution at aa 47 of the NS1 protein.

Although the only consensus change identified in the SLEV 17A strain was a silent change in the 3'-UTR, 17A-9 and 17A-18, both derived from the 17A population, each

<table>
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<tr>
<th>Strain</th>
<th>Vero infectious particles (log(_{10}) p.f.u. ml(^{-1}))</th>
<th>Viral RNA (log(_{10}) genomes ml(^{-1}))</th>
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<tbody>
<tr>
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<tr>
<td>DAE 17A-20</td>
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Neg, negligible.

![Fig. 7. Strain- and cell-specific differences in *in vitro* infectiousness as measured by fluorescent focus assay of unpassaged (WT) SLEV and plaque-purified tick-cell-passaged SLEV (17A-9) 72 h p.i. on both mammalian (Vero) and tick (DAE) cell lines.](image-url)
accrued five substitutions, including two non-synonymous changes. One of these substitutions, A4752G (NS3 gene), was shared by 17A-9 and 17A-18, and resulted in a threonine to alanine substitution within the viral protease, located at NS3 aa 39. In all, five of the 10 substitutions within these two strains were found in the NS3 gene. 17A-9 also accrued an A5919G NS3 substitution, resulting in an isoleucine to valine change within the viral helicase, at NS3 aa 428; whilst the other non-synonymous change in 17A-18 was an A6820G within the NS4A gene, resulting in a lysine to arginine change at NS4A aa 121.

DISCUSSION

RNA viruses that have evolved under conditions of host breadth, such as SLEV and other arboviruses, are likely to be generalists that have an inherent capacity to succeed in novel environments (Turner et al., 2010). We tested the ability of SLEV to replicate in and adapt to a novel invertebrate cell culture environment, DAE cells, and determined both the genetic correlates and phenotypic costs of this experimental evolution. Our results demonstrate, not surprisingly, that SLEV is capable of adapting further to this environment, yet also that the magnitude of adaptation, which is identifiable consistently in competition but not individual replicative kinetics, is variable and relatively subtle as compared with previous studies on SLEV and other related viruses. For instance, following 20 passages of SLEV in C6/36 mosquito cell culture, replicative fitness increases approaching 100-fold were measured by both competition assay and individual growth (Ciota et al., 2007b). Here, a mean increase in relative fitness in tick cells of <10-fold was measured following 15 passages for lineages A and C, and no advantage in individual growth was measured relative to SLEV WT following 19 passages for these lineages. Lineage B, however, displayed a relative fitness increase of almost 1000-fold, as well as ~10-fold increase in peak viral titre when evaluating individual growth on DAE cells. The increased sensitivity of relative fitness as measured by competition assay as compared with replicative fitness as measured by multi-step growth kinetics has been noted in other studies (Holland et al., 1991; Novella et al., 2004). In addition, the variability in fitness gains among lineages is not uncommon (Smith-Tsurkan et al., 2010; Turner & Elena, 2000) and probably reflects the stochasticity of mutation, and therefore evolutionary and adaptive trajectories. The fact that ISE6 cells supported modest SLEV replication following adaptation to DAE cells also demonstrates experimentally how adaptation to an intermediate environment could facilitate a larger host jump, as often noted in other systems (Parrish et al., 2008), but rarely demonstrated experimentally.

The significant adaptation measured in SLEV 15B was associated with only modest consensus genetic change, including single amino acid changes in the envelope and NS1 proteins. This is not surprising, as the majority of experimental evolution studies with arboviruses demonstrate that adaptations are often achieved with few consensus substitutions. For example, mosquito cell adaptation of WNV and SLEV was associated with one (WNV) or two (SLEV) consensus amino acid changes following 19 or 20 passages, respectively (Ciota et al., 2007b). Similar results have also been noted for Dengue virus (Vasilakis et al., 2009), Sindbis virus (Greene et al., 2005) and other arboviruses (Ciota & Kramer, 2010; Novella et al., 2011). Examples have also been documented with natural strains, including the single valine to alanine substitution in the WNV02 strain leading to significantly decreased extrinsic incubation in Culex mosquitoes (Moudy et al., 2007) and the epidemic Chikungunya virus strain associated with increased vector competence in *Aedes albopictus* (Schuffenecker et al., 2006). Taken together, these data demonstrate that although the relative evolutionary stasis of arboviruses in nature is often highlighted, this does not necessarily equate to a lack of phenotypic variability and potential for host adaptation or expansion. It is also important to consider that, although minority substitutions were not identified directly here, limiting dilution experiments with SLEV 17A demonstrate phenotypic and, therefore, genetic intrahost diversity and minority variants may have a significant influence on viral fitness, as previously demonstrated for WNV (Ciota et al., 2007c), vesicular stomatitis virus (Duarte et al., 1994; Novella & Ebendick-Corpus, 2004) and foot-and-mouth-disease virus (Martinez et al., 1991; Ruiz-Jarabo et al., 2002).

The notion that host cycling results in suboptimal host-specific adaptation is often presented as dogma in discussions of arbovirus evolution (Scott et al., 1994; Woelk & Holmes, 2002; Woolhouse et al., 2001). Despite this, evidence for this phenomenon is mixed and although fitness trade-offs certainly at times result from single host adaptation, host-specific evolution may also result in fitness gains or neutral fitness in other hosts (Dearoff et al., 2011; Novella et al., 2007, 2011; Turner et al., 2010). *Previous in vivo* experimental evolution studies with SLEV in mosquitoes and chicks in fact suggest fitness trade-offs in terms of replicative ability and infectivity may generally be lacking for SLEV in its primary transmission cycle (Ciota et al., 2009). The idea of fitness tradeoffs due to cycling extends also to the costs of host range expansion, for which the prediction stands that adaptation to a novel host will often result in decreased fitness in ancestral environments as a consequence of either antagonistic pleiotropy or mutation accumulation (Kassen, 2002; Remold et al., 2008). Although two of the three passage 15 SLEV strains did indeed lose fitness in vertebrate cell culture, we also identified generalist strains with adaptation to mosquito and/or vertebrate cell culture, as well as strains with fitness losses in mosquito cell culture alone. These results lend further support to the idea that evolution to a novel niche could result in a range of phenotypes in alternate environments, particularly for arboviruses, which are presumably well evolved for environmental complexity. How one defines or measures a fitness cost is also important, and variation in these criteria is certainly partially responsible for variability...
within and among studies (Ciota & Kramer, 2010). Here, despite measures of relative fitness being variable among cell lines and lineages, all three lineages did, on average, accrue both decreases in plaque size and infectivity on vertebrate cell culture. These decreases could be important fitness determinants when transmission bottlenecks are narrow (Chao, 1990; Novella, 2004) and may also be associated with other phenotypic variation in vertebrate hosts such as decreased pathogenesis (Ebel et al., 2011). Despite this general trend, characterization of SLEV 17A variants demonstrates that there is diversity on the clonal level within individual strains, with tick-cell-evolved variants ranging from those that clearly accrued a cost in vertebrate cells to those that are neutral or even co-adapted to alternate environments. This diversity, which could be referred to as molecular ‘memory’ (Ruiz-Jarabo et al., 2000) but may be largely stochastic, could provide variants prepared for replication in a broad range of hosts. Previous studies suggest it is the invertebrate environment that is more likely to supply this diversity, and that it is probably maintained by a combination of relaxed purifying selection due to mutational robustness and complementation in co-infected cells (Ciota et al., 2012; Jerzak et al., 2005, 2008). This mutant swarm structure explains how a population of variants could at times retain fitness in multiple environments yet also sacrifice some diversity if environmental complexity is increased. A single passage of mosquito cell-adapted WNV in avian cell culture significantly decreased intra-host diversity in a previous study (Ciota et al., 2007c). Single-host passage in the present study therefore allowed us to identify genetic change important not just in host expansion, but also in host restriction. Whilst those variants isolated from SLEV 17A which produced the most SLEV RNA on DAE cells also generally maintained plaque production and robust growth on Vero cell culture, there were also individual variants identified which maintained the capacity to replicate in tick cell culture but were impaired significantly in terms of vertebrate cell plaque production. Sequencing of the two isolates with the largest discrepancy between RNA produced on DAE cells also generally maintained plaque production and maintained at 33 °C in 5% CO₂. African green monkey kidney cells (Vero; ATCC CCL-81) were grown in minimal essential medium (MEM; Gibco) supplemented with 10% FBS, 2 mM l-glutamine, 1.5 g sodium bicarbonate l⁻¹, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹, and maintained at 37 °C in 5% CO₂. A. albopictus cells (C6/36; ATCC CRL-1660) were grown in MEM supplemented with 10% FBS, 2 mM l-glutamine, 1.5 g sodium bicarbonate l⁻¹, 0.1 mM non-essential amino acids, 100 U penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹, and maintained at 28 °C in 5% CO₂.

SLEV Kern 217.3.1.1 (SLEV WT) was derived from SLEV Kern 217 following three rounds of plaque purification on Vero cells (Ciota et al., 2007b). This strain was isolated originally in 1989 from Culex tarsalis in Kern County, CA, and passaged twice in Vero cells (obtained from Dr William Reisen, University of California at Davis; Kramer & Chandler, 2001).

**Virus passaging.** Three lineages each of SLEV WT were derived by 17 (B) or 19 (A and C) serial passages in DAE cells, using a m.o.i. of 0.1 p.f.u. cell⁻¹, based on Vero cell titres, for each passage. This m.o.i. represented a population size of ~100,000 p.f.u. initiating each new passage. Virus was adsorbed to tick cell monolayers in 12-well plates for 1 h at 33 °C, with frequent rocking. Following adsorption, the inoculum was removed, cells were gently washed with L-15B media, 2.0 ml L-15B media was added to each well and plates were incubated at 33 °C. Medium from each well was harvested at 144 h p.i. and stored in aliquots at −80 °C. Following each passage, viral titre was quantified by plaque assay on Vero cells (Payne et al., 2006) and diluted to produce an m.o.i. of 0.1 p.f.u. cell⁻¹ for each subsequent passage. In addition, viral RNA was isolated (RNeasy; Qiagen) and quantified by qRT-PCR. Forward primer 5'CGTGAGATTGT-CAAGGCAACT, reverse primer 5'TCTCCTGTGTAGGGACCCTT and TaqMan probe 6FAM-ACACGCTCACAACAG-TMGMBNFQ were used for qRT-PCR assays performed on an ABI Prism 7500 sequence detector, using TaqMan One-Step RT-PCR Master Mix (Applied Biosystems), as described previously (Ciota et al., 2007b; Shi et al., 2001). Infectiousness was determined by quantifying the ratio of RNA to p.f.u. and compared throughout passage using one-way ANOVA (GraphPad Prism, version 5.0).

Clonal populations of SLEV 17A were generated by diluting the passaged virus to ~4 p.f.u. (100 μl⁻¹) and then inoculating diluted virus onto multiple wells (n=20) of confluent DAE cells seeded in a 24-well plate. Virus was adsorbed and overlaid as described above. Medium from each well was harvested at 168 h p.i. and stored at −80 °C. Each well was quantified in parallel by plaque assay and qRT-PCR as described above.

**Growth kinetics and viral fitness.** Confluent monolayers of DAE or ISE6 were infected with virus, in duplicate, using 24-well plates, at an m.o.i. of 0.1 p.f.u. cell⁻¹, based on Vero cell titre. After a 1 h absorption period at 33 °C, the inoculum was removed, cells were gently washed, overlaid with 2 ml of maintenance media and the plates were returned to 33 °C. Samples were taken at 24, 48, 72, 96, 120, 144 and 168 h p.i., 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 titres for each time point were calculated and compared using standard two-way t-tests (GraphPad Prism, version 5.0).

The relative fitness of SLEV following tick cell passage was evaluated by competition assays in C6/36, Vero and DAE cells (Ciota et al., 2007b; Holland et al., 1991). Confluent cell monolayers in 24-well plates were infected in triplicate with a 1:1 mixture of control and test virus at a m.o.i. of 0.1 (C636, Vero) or 0.001 (DAE) p.f.u. cell⁻¹, based on Vero cell titre. A SLEV mAb-resistant mutant (MARM), demonstrated previously to have similar fitness to SLEV WT (Ciota

**METHODS**

**Cells and viruses.** Hard tick cell lines (kindly provided by Timothy Kurtti) *I. scapularis* (ISE6) and *D. andersoni* (DAE) were grown in L-15B medium, pH 7.0, containing 5% tryptose phosphate broth, 10% FBS and 0.1% bovine lipoprotein cholesterol concentrate, and maintained at 33 °C in 5% CO₂.

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et al., 2007b), was confirmed to have similar fitness in DAE cells (data not shown) and therefore served as a surrogate WT control. The test viruses consisted of the SLEV strains passed in DAE cells. After a 60 min absorption period at 28 °C (C6/36), 37 (Vero) or 33 (DAE) °C, the infected monolayers were washed three times, overlaid with 1.5 ml maintenance medium and incubated at the temperatures used for the infection. Medium from the infected cultures was harvested at 72 (Vero), 96 (C6/36) or 144 (DAE) h p.i., based on peak titre of previous growth curves. Harvested samples were diluted 1:10 in growth medium supplemented with 20 % FBS and frozen at −80 °C for later titration, in duplicate, on Vero cells. The competition assays were evaluated by measuring the amount of control and test virus present in the initial input mixture and at the end of the competition, by duplicate plaque assay on Vero cells, in the presence and absence of highly neutralizing mAb in the agar overlay. The quantity of test virus was obtained by subtracting the titre in the presence of mAb (MARM control titre) from the titre without mAb (total titre) and the test/control virus ratio was determined for the competition. Final relative fitness values were calculated for each assay by normalizing the output ratios to both the quantified ratio of the initial input and the ratio of SLEV WT to SLEV MARM, thereby providing a numerical representative of change in ratio of passages strains relative to SLEV WT (Ciota et al., 2012; Duarte et al., 1992; Martinez et al., 1991). Mean ± SD for triplicate values were compared with a hypothetical fitness value of 0 (neutral fitness) using a one-sample t-test (GraphPad Prism, version 5.0).

**Fluorescent focus assay.** Eight-well Lab-Tek II Chamber Slides (Nalge Nunc International) were seeded with Vero or DAE cells at a density of 2.5 × 10^4 cells well^−1^ and incubated for 24 h at 37 °C (Vero) or 37 °C (DAE) in 5% CO₂ to produce a confluent monolayer. The cell monolayers were inoculated with 10-fold serial dilutions of virus in a final volume of 0.05 ml; virus was adsorbed to the cells for 1 h at 37 (Vero) or 33 (DAE) °C, with gentle rocking every 15 min. An overlay of MEM, 5% FBS and 0.8% carboxy methyl cellulose (ICN Biomedicals; Vero) or L-15B (DAE) was added at the conclusion of adsorption. The infected monolayer was incubated at 37 (Vero) or 33 (DAE) °C in 5% CO₂. After 48 (Vero) or 72 (DAE) h of infection, the overlay medium was removed and the cells were washed with cold PBS. The PBS was removed after 5 min, and cells were then fixed for 10 min with ice-cold absolute methanol (Sigma-Aldrich) and again washed in PBS for 5 min. Slides were placed in a moist chamber and in SLEV antibody (MHIAF 6B5A-2; WCL&R Laboratories). Fluorescently labelled cells were evaluated using a Zeiss Axiostar 25 microscope, equipped with a Fluar ×10 objective and FITC filter. Images were photographed with a Zeiss Axioscan MRC digital camera and Axiovision software. The area of each fluorescent focus was measured (mm^2^) using Axiovision software. The focus size (mean ± SEM) was calculated for each lineage, and compared with Mann–Whitney U and linear regression tests (GraphPad Prism, Version 5.0).

**Full-genome sequencing.** SLEV RNA was extracted from DAE-derived SLEV using RNasy (Qiagen) according to the manufacturer’s protocol and sequencing was carried out as described previously (Ciota et al., 2007b). One-step RT-PCR (Qiagen) with nine overlapping primer sets was carried out to generate PCR products for sequencing. RT reactions were completed at 50 °C for 30 min, followed by inactivation of the transcriptase at 95 °C for 15 min. Amplification was then carried out for 40 cycles at 94 °C for 20 s, 55 °C for 30 s and 72 °C for 2 min, with final elongation at 72 °C for 10 min. PCR products were visualized on a 1.5 % gel and bands were gel-extracted using the Qiagen gel extraction protocol. Sequencing was performed at the Wadsworth Center Applied Genomics Core using an ABI 3700 automated sequencer (Applied Biosystems) with a minimum of twofold redundancy. Sequences were compiled and edited using the DNASTAR software package.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


