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Genomic stability of La Crosse virus during vertical and horizontal transmission

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Summary. We have used ribonuclease T1 oligonucleotide fingerprint analysis to study genomic stability of La Crosse virus (*Bunyaviridae*) during vertical and horizontal transmission in the laboratory. No RNA genomic changes were detected in vertebrate cell culture-propagated virus isolated (following ingestion and replication) from the natural host, *Aedes triseriatus*. Genomic changes were not detected during transovarial passage of the virus through two generations of mosquitoes, nor were changes detected in the genomes of virus isolated from suckling mice that had been fed upon by second generation transovarially-infected mosquitoes. These results demonstrate that despite the well-documented phenomena of rapid nucleotide change in RNA virus genomes under various conditions, the La Crosse virus genome can remain stable during transovarial transmission in the insect host and during transfer between the insect and vertebrate hosts. The evolutionary implications of these results are discussed.

Introduction

La Crosse (LAC) virus is a member of the California serogroup consisting of 13 viruses in the genus *Bunyavirus*, family *Bunyaviridae*. These viruses are characterized by 3-segmented, single-stranded, negative-sense RNA genomes and a host-derived lipid envelope bearing 2 coat glycoproteins designated G1 and G2 [22]. The virions are approximately 90 nm in diameter. The 3 viral RNA segments are designated as large (L), medium (M), and small (S). The L RNA is approximately 7–8 kilobases (KB) and encodes the viral RNA-directed RNA polymerase [11]. The M RNA is approximately 4.5 KB and encodes G1 and G2 and 1 or more nonstructural proteins in the same reading frame [12, 13]. The S RNA, approximately 0.9 KB, encodes the nucleocapsid protein and, in an overlapping reading frame, a nonstructural protein of unknown function [5].

LAC virus is distributed throughout the mid-western United States and is maintained in nature by a cycle involving a woodland mosquito, *Aedes triseriatus,* which vectors the virus to the principal vertebrate hosts, chipmunks and squirrels. When humans impinge upon this cycle, infection with LAC virus can result. With the exception of those years when St. Louis encephalitis virus epidemics occur, LAC encephalitis is "probably the most important mosquito-borne disease in the United States" [17].

In the LAC virus cycle the genetic information of the virus must be replicated and expressed in the distinctly different biological systems of the invertebrate and vertebrate hosts. Viral replication in vertebrate cells causes cytopathologic effects (CPE), and LAC virus infection of children results in significant morbidity and infrequent mortality [19]. In contrast, viral replication in mosquito cells does not cause CPE, and no major negative effects on the mosquito host have been reported. Peristent infection of the mosquito host, and venereal (horizontal) and transovarial (vertical) transmission of the virus have been demonstrated [28, 31]. The virus overwinters in diapaused eggs of the mosquito host [32].

In view of the differences in the nature of the LAC virus infection in the vertebrate and invertebrate hosts and the high mutability and rapid evolutionary potential of RNA viral genomes [20, 23, 24], one might expect that environmental differences faced by the virus in the two host types could select for different genotype distributions in the virus population. Such a phenomenon could have important epidemiological implications including changes in transmission potential, virulence, host tropisms, and ability to evade prevalent immunologic barriers in vertebrate hosts [2]. We have used the technique of RNase T1 oligonucleotide fingerprinting (ONF) to study the possibility that the LAC virus genome may evolve when passaged between and within vertebrate and invertebrate hosts under laboratory conditions.

Materials and methods

Virus

LAC virus, originally isolated from a fatal human encephalitis case [29] and serially passaged many times in BHK-21 cell culture monolayers, was further passaged 1 time in mice and 1 time in BHK-21 cell monolayers. The virus was then plaque-purified 3 times on BHK-21 cell monolayers, followed by a final low multiplicity of infection (MOI) passage in BHK-21 cell monolayers to prepare inoculum stock.

Mosquitoes

Aedes triseriatus mosquitoes were collected in La Crosse, Wisconsin in 1981. Virus and mosquitoes were kindly provided by Dr. Wayne Thompson, University of Wisconsin. The mosquito colony has been maintained at Colorado State University for 7 years under the following conditions: photoperiod 16 h light/8 h dark, 60–80% relative humidity, 23–25 °C. Adults were maintained on sugar cubes, raisins, apples and water. Larvae were fed TetraMin (Tetra Works, Melle, Federal Republic of Germany).

Cell culture

BHK-21 cells were obtained from the American Type Culture Collection and were grown at 37 °C, 8% carbon dioxide in DMEM media (Gibco) supplemented with 10% bovine calf serum (Hyclone). Medium for production of ^{32}P-labelled virus was phosphate-free DMEM with 25 Mm HEPES (Hazleton) supplemented with 2% dialyzed fetal bovine serum (Hyclone) and ^{32}P-orthophosphate (ICN).

Artificial bloodmeal preparation and infection of mosquitoes

Uninfected female mosquitoes were allowed to feed on artificial bloodmeals containing plaque-purified LAC virus. The bloodmeals were prepared by growing virus in BHK-21 cell culture monolayers infected at a MOI of 0.01. When cells exhibited 3 + CPE they were scraped into the media and transferred to a centrifuge tube. Following a 10 min, 200 × **g** centrifugation, all but 2 ml of the supernatant was poured off. The cells were resuspended and gently mixed with 2 mls each of washed human red blood cells, heat-denaturated fetal bovine serum and 10% sucrose. The mixture was warmed to 37 °C and was offered to mosquitoes as droplets placed on the cage screening. The virus titre was 7.1 $\log_{10}$ $TCID_{50}$/ml. The infection rate of mosquitoes taking bloodmeals was determined by immunofluorescent examination of a leg [1] following 14 days extrinsic incubation.

Growth of virus isolates and preparation of viral RNA for oligonucleotide fingerprinting

All mosquitoes and mouse brains stored at −70 °C were homogenized in 100 µl DMEM. The homogenates were passed through 0.2 µm syringe filters. A 1 ml volume of a 100-fold dilution of each filtrate was used to infect a 175 cm^2 tissue culture flask of confluent BHK-21 cells. When the cells exhibited 3+ CPE the culture media was collected and clarified by a 10 min, 1,000 × **g** centrifugation and was stored as a virus stock at −70 °C. These stocks were used to infect BHK-21 cell monolayers at MOI values of 0.1–1.0 in order to prepare ^{32}P-labelled viral RNA for ONF. We have chosen BHK-21 cells in preference to a mosquito cell line because of technical difficulties in obtaining sufficient ^{32}P-labelled virus for ONF from mosquito cell cultures. Following infection, the cell cultures were maintained at 33 °C for 24–36 h in phosphate-free DMEM supplemented with 2% dialyzed fetal bovine serum and ^{32}P-orthophosphate at 10 µCi/ml. The cell culture medium was collected and clarified by a 30 min, 10,000 rpm centrifugation in a Sorvall SS-34 rotor at 4 °C. The supernatant was loaded onto 7 ml cushions of 30% w/w glycerol in TE buffer (10 mM Tris/HCL, 1 mM EDTA, pH 8.0 at 4 °C). The virus was pelleted at 4 °C for 2½ h at 24,000 rpm or for 15 h at 15,000 rpm in a Beckman SW-28 rotor. The pellets were resuspended for 1 h on ice in 0.4 ml each of TE buffer and 25 µl of 5 M NaCl. Three mls of resuspended virus was loaded onto a linear gradient prepared with 5 ml of 30% w/w glycerol in TE buffer and 4.5 ml of 50% w/w sodium potassium tartrate in TE buffer. The gradients were centrifuged at 4 °C for 2–4 h at 35,000 rpm or 15 h at 18,000 rpm in a Beckman SW-41 rotor. Visible virus bands (present about halfway down the gradients) were collected and diluted to 10 ml final volume with TE buffer. The diluted virus was loaded onto 2 ml cushions of 30% w/w glycerol in TE buffer and was pelleted for 2 h at 4 °C at 35,000 rpm in the SW-41 rotor. The viral pellets were resuspended in 0.3 ml TE buffer with 2% SDS at room temperature, and were extracted twice with equal volumes of phenol saturated with TE buffer, and once with 24 : 1 v/v chloroform/isoamyl alcohol. The aqueous phase, containing viral RNA, was ethanol-precipitated overnight at −20 °C. The RNA was pelleted, resuspended in 40 µl TE buffer/2% SDS, and the viral L, M, and S RNA segments were separated by electrophoresis in 1% low-melting point agarose gels buffered with TBE at 5 V/cm for 6 h. The position of L, M, and S RNA in the gels was determined by autoradiography. Sections of gel containing the L, M, and S RNA were excised, melted at 65 °C and extracted

twice with equal volumes of phenol and once with chloroform/isoamyl alcohol. The aqueous phases were ethanol-precipitated with 10 µg tRNA as carrier.

RNase T1 oligonucleotide fingerprinting

The viral RNAs were pelleted, resuspended in 50 µl TE buffer and digested for 1 h at 37 °C with 100 units of RNase T1 (BRL). The digests were phenol and chloroform extracted as above and ethanol precipitated overnight at −20 °C. The viral RNA was pelleted, resuspended in 6 µl 6 M urea and subjected to two-dimensional polyacrylamide gel electrophoresis essentially as described earlier [8] with modifications [18]. Autoradiography of the second dimension gel was performed with Kodak X-OMAT AR or Konica Type A X-ray film at −70 °C with intensificaton screens.

Results

The passage history is diagrammed in Fig. 1. A group of mosquitoes infected with plaque-purified virus by artificial bloodmeal was allowed to feed a second time on uninfected adult mice and oviposit. One of these mosquitoes (Original Female) was triturated and stored at −70 °C. Her eggs were hatched and reared, and the adult F1 progeny were tested for virus infection. The transovarially-infected females were allowed to feed on uninfected adult mice and oviposit. One of these females (F1 Daughter #8) was triturated and stored at −70 °C. Her eggs were hatched and reared, and the adult F2 progeny were tested for virus infection. The transovarially-infected F2 progeny were allowed to blood-feed separately on individual uninfected suckling mice (Balb C, Charles River Laboratories, Willington, MA) before the mosquitoes were triturated and stored at −70 °C. Mosquitoes F2 Daughter #2 and F2 Daughter #3 were from this group. The suckling mice were held until ill (symptoms included shaking, inability to remain upright, cessation of nursing, and dehydration), and brains

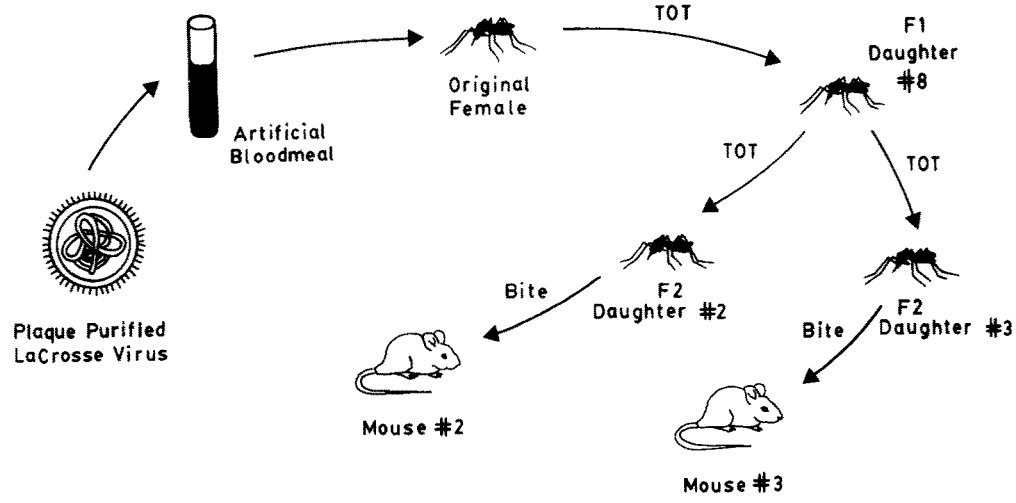

Fig. 1. Passage history of La Crosse virus. Plaque-purified virus was passaged through this series of hosts. Virus was isolated from each host and subjected to oligonucleotide fingerprint analysis. *TOT* Transovarial transmission, *Bite* transmission by bite of an infected mosquito

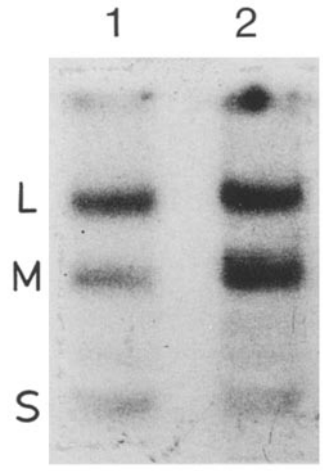

Fig. 2. Autoradiogram of two preparations of La Crosse virus RNA separated in a 1% low-melting point agarose gel. *1* Virus isolated from the infective bloodmeal. *2* Original plaque-purified virus. *L* Large RNA, *M* medium RNA, *S* small RNA

were then removed, homogenized and stored at −70 °C. Mouse #2 and Mouse #3 were infected by bite of mosquitoes F 2 Daughter #2 and #3 respectively.

After 2 weeks extrinsic incubation, 65/83 or 78% of mosquitoes ingesting bloodmeals tested positive for virus infection. This compared favorably with the 95% infection rate for a second group of mosquitoes intrathoracically inoculated with virus. The F 1 generation progeny of the Original Female had a filial infection rate of 19% and the F 2 progeny of F 1 Daughter #8 had a rate of 46%.

An autoradiogram of LAC virus RNA prepared as described and electrophoresed in a low-melting point agarose gel is shown in Fig. 2. Only 3 major bands are present, corresponding to the viral L, M, and S segments. The absence of other bands demonstrates that these viral RNA preparations were relatively free of cellular contaminants and were suitable for ONF.

The L, M, and S segment oligonucleotide fingerprints of the original plaque purified LAC virus and virus isolated from F 2 Daughter #2 and Mouse #2 are shown in Fig. 3. There were no detectable changes in these fingerprint patterns of the three viral RNA segments of LAC virus isolated from any host in the passage history diagrammed in Fig. 1. From 1–4 replicate fingerprints were obtained for each RNA segment of virus isolated from any particular host.

Discussion

We conclude that no detectable nucleotide changes occurred in the LAC virus genome upon passage of vertebrate cell culture-propagated virus in the natural mosquito host, following oral infection. Furthermore, no nucleotide changes in the viral genome occurred during 2 generations of transovarial transmission

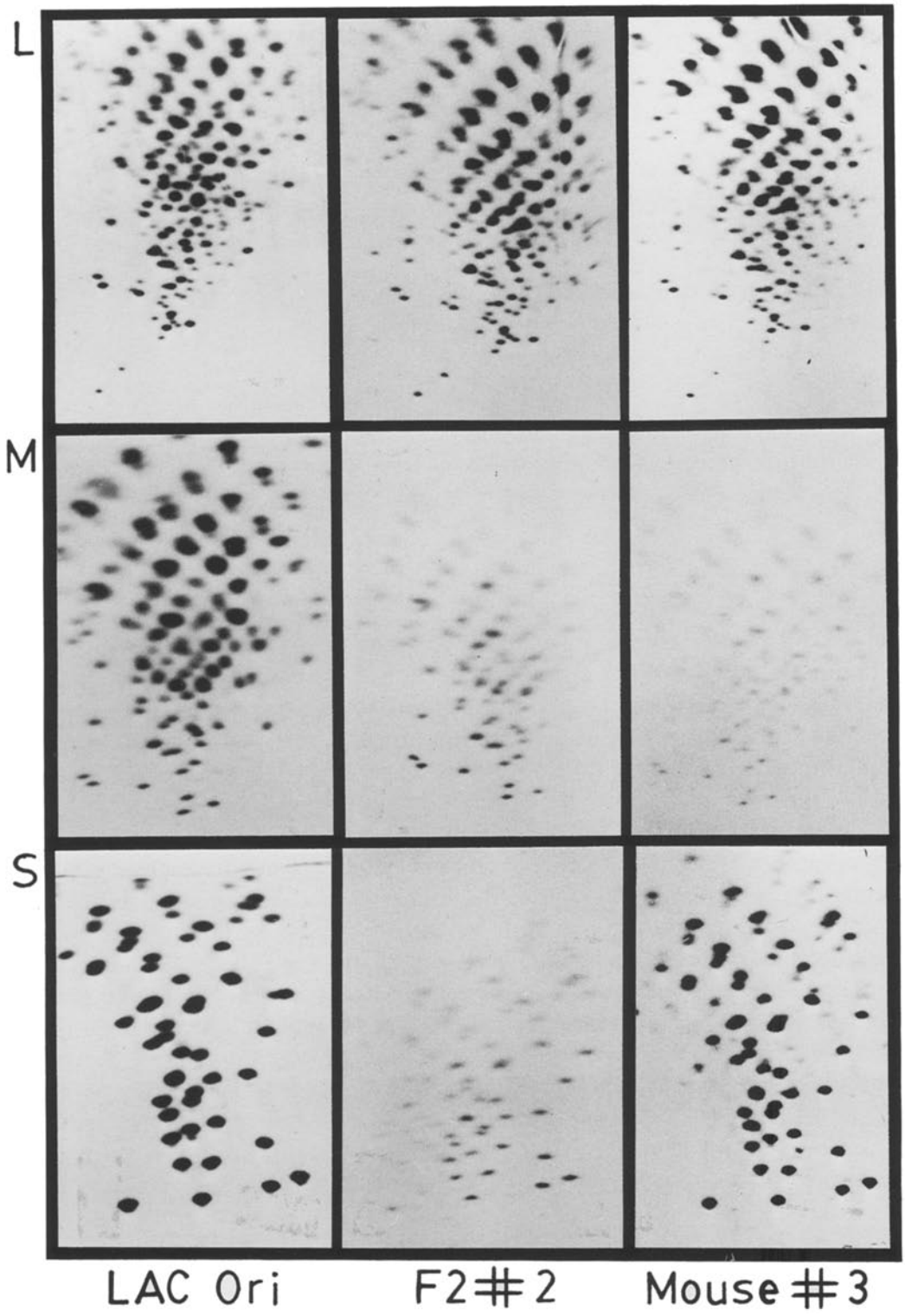

Fig. 3. Oligonucleotide fingerprint autoradiograms of original plaque-purified La Crosse virus (*LAC Ori*) and of virus isolated from F 2 Daughter # (*F 2# 2*) and Mouse # 3. *L* Large RNA, *M* medium RNA, *S* small RNA

(TOT) in the mosquito host, or upon transmission to and replication in a vertebrate host by bite of a second generation transovarially infected mosquito.

It is possible that a small number of nucleotide changes occurred and escaped detection due to the generally accepted fact that ONF directly samples only 10–30% of the viral genome, depending on size and number of segments. Since the L, M, and S segments were independently analyzed we have probably attained the upper end of the sampling range. It is also possible that our failure to detect changes in nucleotide sequence is due to use of long-passaged virus, and/or BHK-21 cells rather than mosquito cells to prepare viral RNA for fingerprinting. However, these are standard methods in common use for ONF analysis of viral genomes.

There is growing interest in the concept of RNA virus populations in existence as heterogenous mixtures of variant genomes, that is, as "quasispecies" [9], with extremely rapid evolutionary potential exceeding that of their eucaryotic hosts by as much as a million-fold [20, 21, 23, 24]. A large body of evidence underlies the modern concept of an RNA virus quasispecies population which exists in a stable equilibrium dominated by the most genetically fit variants [23]. When environmental conditions change, such as in transfer to a new host, population disequilibrium and rapid continuous evolution of the viral genome may occur.

Evidence indicates that this genomic plasticity may apply to LAC virus. Some 30 field isolates of LAC virus obtained from several geographic locations at the same or at different times were subjected to ONF analysis [10, 16]. None of the isolates yielded identical fingerprints, although some were obviously closely related. We have fingerprinted 5 different laboratory stocks of LAC virus and have found only minor differences among them (data not shown). Our fingerprint studies of 3 laboratory strains of the Montana isolate of Snowshoe Hare virus have shown only minor differences among the three. In contrast, 2 Snowshoe Hare virus isolates from mosquitoes in the Yukon Territory, Canada have major differences [Clerx et al., submitted]. These results indicate genetic variation among various geographically isolated populations of LAC virus and its close relative, Snowshoe hare virus. The LAC virus genome can respond to changes in selective pressure, as shown by the fact that a monoclonal antibody-selected variant exhibiting decreased cell fusion ability, loss of neuroinvasiveness in mice, and decreased infectivity in mosquitoes reverted following recovery from an infected mosquito and serial passage in BHK-21 cells [14, 25]. Tesh and Gubler [26] have demonstrated the presence of small plaque variants in populations of transovarially passaged LAC virus, indicating a heterogenous virus population. Furthermore, the spontaneous mutation rate of Snowshoe Hare virus is 1–2% [4].

Despite these potential indications of genomic plasticity in LAC virus, we have demonstrated that the virus does not appear to undergo rapid evolution during TOT in the mosquito host or during horizontal transmission from mosquito host to vertebrate host under laboratory conditions. Bilsel et al. [3] have

recently reported that Toscana virus (*Phlebovirus, Bunyaviridae*), serially passaged by TOT over a period of 2 years through 12 generations of the natural sandfly host, underwent no detectable genomic change as determined by ONF. Our extension of these results to LAC virus in mosquitoes allows us to speculate that genomic stability of the viral genome during TOT may be a general phenomenon among *Bunyaviridae* and perhaps among other arboviruses.

The rate of evolution of Ross River virus (a mosquito-borne positive-sense RNA virus in the family *Alphaviridae*) has recently been examined by Burness et al. [6] during the course of a 10-month outbreak of epidemic polyarthritis in a non-immune island population of humans. With use of direct genomic sequencing of 2 areas of the virus genome totaling 1.6 KB, and previously shown to be variable among different wild isolates of the virus, they demonstrated that only 1 nucleotide substitution occurred in the virus isolates examined over the course of the epidemic.

Three different arboviruses, LAC, Toscana, and Ross River, have now been shown to undergo no or very low rates of evolution under conditions in which they might reasonably be expected to evolve at higher rates, and despite the fact that wild isolates of the viruses do exhibit genetic diversity. Furthermore, ONF analysis of various Alphaviruses isolated over a period of 25 years and over wide geographic ranges have demonstrated remarkable similarity among isolates of any particular virus [for review, see 2]. Taken together, these results may be interpreted to indicate low rates of evolution among arboviruses relative to those of non-arthropod-borne RNA viruses.

The apparent low rate of evolution among arboviruses thus requires explanation. It is unlikely that it is due to inherent genetic stability of arboviruses since almost all RNA viruses show similar rates of spontaneous mutation. Selective pressures unique to the arbovirus cycle must therefore underlie the low rate of evolution. The fact that arboviruses must be able to infect and then replicate and express their genomes in both vertebrates and invertebrates may itself be a factor in their apparent genetic stability. It has been suggested that transmission between invertebrate and vertebrate hosts may create population disequilibrium in arboviruses leading to generation of new virus populations with different spectrums of variants [15]. However, it is also possible that molecular constraints placed on the virus by such disparate hosts may be a powerful selective force for genomic stability by limiting the spectrum of viable and competitive variants in a cycling arbovirus population. The recent demonstration that a translational requirement for complete La Crosse virus S segment mRNA synthesis is operative in vertebrate cell culture, but not in mosquito cell culture [Raju and Kolakofsky, pers. comm.], underscores the probable involvement of intra-cellular molecular events in the biological differences seen in arbovirus infections of vertebrate and invertebrate hosts.

During TOT many rounds of virus replication must occur in successive related generations of mosquitoes. If the laboratory observations of LAC and Toscana virus genomic stability during TOT are true of the situation in nature,

then it is possible that this mechanism could confer a low rate of evolution on the virus. If one accepts the premise that certain arboviruses, including LAC, may be principally maintained in nature within the invertebrate host by transovarial and venereal transmission [27, 30] then a role for these transmission mechanisms in a low rate of viral evolution becomes more plausible. The genetic variability seen among wild isolates of LAC virus might be partly explained by the existence of virus populations maintained by TOT in reproductively isolated mosquito populations. When such a virus population is transmitted between invertebrate and vertebrate hosts within the arbovirus cycle, dominant variants are likely to be conserved by physical and biological limitations imposed by small bloodmeal and injected saliva volumes and low virus titers [6, 33]. However, it must be noted that vertebrates possess immune systems with the demonstrated ability to select RNA virus variants during persistent infection [7].

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