



Research article

Potential of Ilhéus virus to emerge

Kenneth S. Plante^{a,b,1}, Jessica A. Plante^{a,b,1}, Sasha R. Azar^{c,d}, Divya P. Shinde^{a,b}, Dionna Scharton^{a,b}, Alice F. Versiani^c, Natalia Ingrid Oliveira da Silva^c, Taylor Strange^c, Livia Sacchetto^e, Eric B. Fokam^f, Shannan L. Rossi^{c,g,i}, Scott C. Weaver^{a,b,g}, Rafael E. Marques^h, Mauricio L. Nogueira^{c,e,h}, Nikos Vasilakis^{c,g,i,*}

^a World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston, TX, 77555, USA

^b Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, 77555, USA

^c Department of Pathology, University of Texas Medical Branch, Galveston, TX, 77555, USA

^d Center for Tissue Engineering, Department of Surgery, Houston Methodist Research Institute, Houston Methodist Hospital, Houston, TX, 77030, USA

^e Laboratório de Pesquisas em Virologia, Departamento de Doenças Dermatológicas, Infecciosas e Parasitárias, Faculdade de Medicina de São José do Rio Preto, São José do Rio Preto, 15090-000, SP, Brazil

^f Laboratory for Biodiversity and Conservation Biology, Department of Animal Biology and Conservation, Faculty of Science, University of Buea, Buea, Cameroon

^g Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX, 77555, USA

^h Brazilian Biosciences National Laboratory, Brazilian Center for Research in Energy and Materials, Campinas, 13083-100, SP, Brazil

ⁱ Center for Vector-Borne and Zoonotic Diseases, University of Texas Medical Branch, Galveston, TX, 77555, USA

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ABSTRACT

Ilhéus virus (ILHV)(*Flaviviridae:Orthoflavivirus*) is an arthropod-borne virus (arbovirus) endemic to Central and South America and the Caribbean. First isolated in 1944, most of our knowledge derives from surveillance and seroprevalence studies. These efforts have detected ILHV in a broad range of mosquito and vertebrate species, including humans, but laboratory investigations of pathogenesis and vector competence have been lacking. Here, we develop an immune intact murine model with several ages and routes of administration. Our model closely recapitulates human neuroinvasive disease with ILHV strain- and mouse age-specific virulence, as well as a uniformly lethal *Ifnar*^{-/-} A129 immunocompromised model. Replication kinetics in several vertebrate and invertebrate cell lines demonstrate that ILHV is capable of replicating to high titers in a wide variety of potential host and vector species. Lastly, vector competence studies provide strong evidence for efficient infection of and potential transmission by *Aedes* species mosquitoes, despite ILHV's phylogenetically clustering with *Culex* vectored flaviviruses, suggesting ILHV is poised for emergence in the neotropics.

1. Introduction

Ilhéus virus (ILHV) is a neuroinvasive neglected tropical pathogen in the Ntaya antigenic group in the family *Flaviviridae*, genus

* Corresponding author. Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA.

E-mail address: nivasila@utmb.edu (N. Vasilakis).

¹ KSP and JAP contributed equally to this work.

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Orthoflavivirus, and has the potential to pose a threat to human health. The virus was originally isolated in 1944 from *Psorophora* and *Aedes* spp. mosquitoes in Ilhéus, State of Bahia, Brazil [1,2]. In the following years, the virus was detected in several additional mosquito genera., including *Culex*, *Coquillettidia*, *Haemagogus*, *Ochlerotatus*, *Sabethes*, and *Trichoprosopon* [3–9]. The virus has also been detected by viral isolation or PCR-based methods in various bird species and humans throughout Central and South America and the Caribbean [10–17]. Based on these detections, as well as on its phylogenetic relationship to better characterized flaviviruses such as West Nile virus (WNV) for which the transmission cycle has been well documented [18–23], ILHV is assumed to be maintained in a transmission cycle between *Culex* spp. mosquitoes and birds. However, serologic surveys indicate that ILHV may infect a wide range of animals including non-human primates, horses, coatis, rodents, bats, tortoises, and sloths [7,16,24–38]. Whether these diverse species represent potential amplifying hosts or dead-end hosts is unclear. This broad circulation in the neotropics, accompanied by human expansion into previously unpopulated or sparsely populated regions, makes ILHV a threat for spillover events into human populations.

To date, ILHV has not been associated with any large epidemics, although a close relative, Rocio virus (ROCV), was responsible for an outbreak in 1976 that infected well over 1000 people with a 13% case fatality rate [39]. ILHV is the etiologic agent of Ilhéus fever, and has been sporadically reported in Bolivia, Brazil, Columbia, Ecuador, French Guiana, Panama, and Trinidad and Tobago [11–15, 38,40]. Ilhéus fever generally presents as an undifferentiated febrile illness that can progress to neurological involvement [13,14,38, 40–43]. In 2017, a retrospective study of patients with central nervous system impairment in São Paulo, Brazil detected ILHV genomic material in the cerebrospinal fluid of an elderly male patient with right hemiplegia, dysarthria, aphasia, and intraparenchymal hemorrhage [44]. This is believed to be the first report of fatal ILHV infection in a human.

Herein, we leveraged the extensive collection of the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) to better understand ILHV's potential to emerge and cause epidemics. Multiple strains of ILHV were characterized to represent the maximum degree of geographic, temporal, and genetic diversity within the viral species [45]. Replication kinetics assessed in immortalized cell lines from multiple mammalian and mosquito spp. supports the serological evidence for ILHV circulation in a wide array of host and vector species. We established an immune-intact murine model which mimics the neuroinvasive phenotype of ILHV in humans in an age-dependent manner. Additionally, we established an immunocompromised murine model with high viremia to facilitate vector competence studies and universal lethality to facilitate the stringent testing of therapeutics and vaccines. Our vector competence studies indicate that, although ILHV is a neurotropic flavivirus that utilizes birds as primary amplification and reservoir hosts and groups phylogenetically with other *Culex*-vectored flaviviruses [46,47], the *Aedes* spp. mosquitoes were far more susceptible to infection in a laboratory setting. These studies increase our understanding of this neglected tropical pathogen and create a pathway for further studies of this potential emergent and underreported threat to human health.

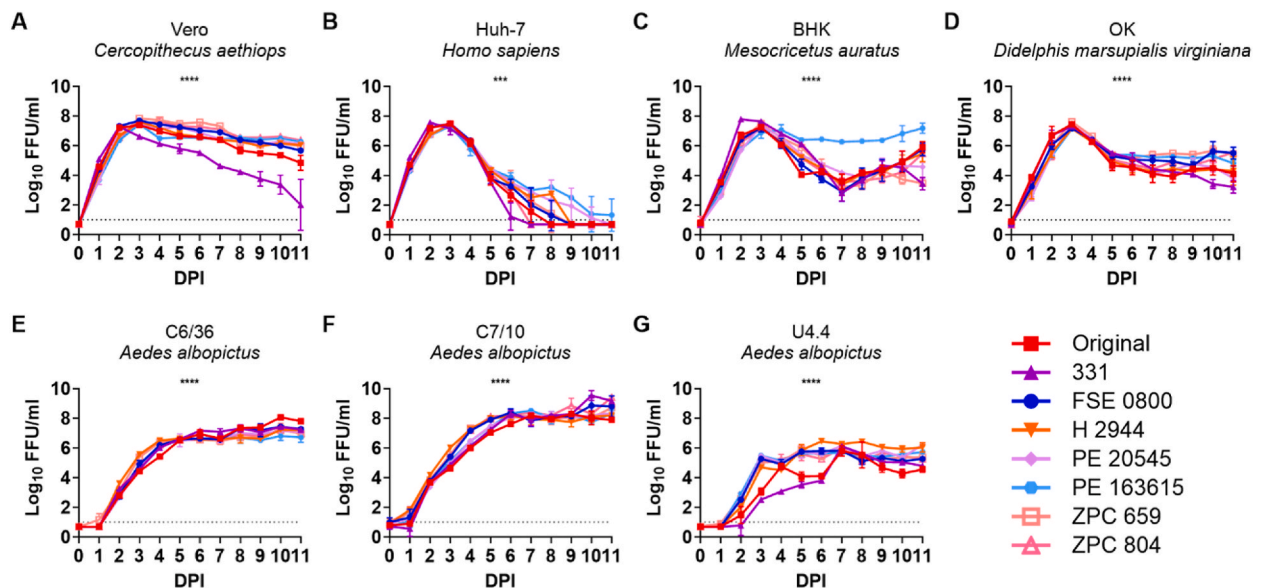


Fig. 1. ILHV replicates to high titers in cultured cells from a variety of mammalian and mosquito species. (A) Vero, (B) Huh-7, (C) BHK, (D) OK, (E) C6/36, (F) C7/10, and (G) U4.4 cells were infected with eight strains of ILHV at a multiplicity of infection of 0.01. Samples were collected daily for 11 DPI, and viral titer was determined by focus forming assay in Vero cells. Data is representative of three independent replicates from a single experiment. Colors represent the ILHV strain, symbols represent the mean, and error bars represent the standard deviation. Dotted lines represent the lower limit of detection. Samples with no detectable ILHV are represented as half the lower limit of detection. Log₁₀-transformed titers were analyzed by two-way ANOVA; the significance of overall ILHV strain-based variation is presented within each panel, and the daily strain by strain comparisons with Tukey's multiple comparisons tests are presented in Table S2 ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

2. Methods

2.1. Cell lines

Vero (CCL-81, ATCC, Manassas, VA) and BHK (CCL-10, ATCC, Manassas, VA) cells were grown in Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (FBS, R&D Systems, Flowery Branch, GA) and 1% penicillin-streptomycin solution (10^4 U/ml and 10^4 µg/ml solution, respectively) (PenStrep, Gibco, Grand Island, NY). Huh-7 (JTC-39, Japanese Health Sciences Foundation, Osaka, Japan) and C7/10 (WRCEVA, originally deposited by Victor Stollar, Rutgers Medical School) cells were grown in DMEM supplemented with 10% FBS, 1% PenStrep, 1% MEM non-essential amino acid solution (Sigma, St Louis, MO), and 1% sodium pyruvate solution (Sigma, St Louis, MO). C6/36 cells (CRL-1660, ATCC, Manassas, VA) were grown in DMEM supplemented with 10% FBS, 1% PenStrep, and 10% tryptose phosphate broth solution (TPB, Sigma, St Louis, MO). U4.4 cells (WRCEVA, originally deposited by Rachel Hernandez and Dennis Brown, NCSU) were grown in Mitsuhashi and Maramorosch Insect Medium (HiMedia Laboratories, Kennett Square, PA), supplemented with 20% FBS, 1% PenStrep, 5% TPB, and 0.15% sodium bicarbonate (7.5% w/v solution) (Corning, Manassas, VA). OK cells (CRL-1840, ATCC, Manassas, VA) were grown in minimum essential media (MEM, Gibco, Grand Island, NY), supplemented with 10% FBS and 1% PenStrep. Vero, BHK, Huh-7, and OK cells were maintained at 37 °C with 5% CO₂. C6/36, C7/10, and U4.4 cells were maintained at 28 °C with 5% CO₂.

2.2. Viruses

Eight strains of ILHV (Table S1) were obtained from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch at Galveston, TX. Viruses were passaged once in Vero cells to generate working stocks. Stocks utilized in OID_{50} experiments underwent one additional passage in C7/10 cells, which yielded higher titer stocks than Vero cells (see Fig. 1 and Table S2) and were required to span a sufficient bloodmeal titer range for OID_{50} determination.

2.3. Virus quantification

ILHV was quantified via the focus forming assay. Briefly, virus was serially diluted in Vero maintenance media (MEM supplemented with 2% FBS and 1% each of GlutaMax (Gibco, Grand Island, NY), PenStrep, and sodium bicarbonate and inoculated onto 12-well plates of Vero cells. Virus was allowed to infect for 1 h in a 37 °C, 5% CO₂ incubator. Following this incubation, an overlay of Opti-MEM (Gibco, Grand Island, NY) supplemented with 2% FBS, 1% PenStrep, and 1% carboxymethylcellulose (Sigma, St Louis, MO) was added to the wells and the plates were returned to the 37 °C, 5% CO₂ incubator. After three days, the plates were fixed with 10% buffered formalin. Fixed plates were blocked with 5% non-fat milk in phosphate buffered saline (PBS, Sigma, St Louis, MO). Anti-ILHV mouse immune ascitic fluid (WRCEVA, Galveston, TX) was diluted 1:1000 in blocking buffer and allowed to bind to the fixed monolayers. Plates were washed with PBS, and HRP-conjugated goat anti-mouse IgG secondary antibody (KPL, Gaithersburg, MD) was diluted 1:2000 in blocking buffer and allowed to bind. Plates were subjected to a final set of washes and developed using KPL TrueBlue peroxidase substrate (SeraCare, Milford, MA).

2.4. Replication kinetics

Comparative multi-step growth curves of all available ILHV strains were performed in triplicate on Vero, BHK, Huh-7, OK, C6/36, C7/10, and U4.4 cells. Cells were seeded one day prior to infection in 12-well plates. Cell lines were infected with ILHV strains at multiplicity of infection of 0.01 in their respective cell culture media and maintained for 1 h at the appropriate temperature for the cell line (28 °C for mosquito cells, 37 °C for vertebrate cells) with 5% CO₂. Following the 1-h incubation, cells were washed with PBS and fresh media was added with the FBS content reduced to 2%. Aliquots were harvested daily through 11 DPI, at which time they were clarified by low-speed centrifugation and stored at -80 °C. Monolayer integrity was documented every other day from days 2–10 post-infection utilizing a CKX53 inverted microscope (Olympus, Waltham, MA) with the 10× CACHN-IPC objective lens (Olympus, Waltham MA) and an attached EP50 digital camera (Olympus, Waltham, MA). Viral titer was determined by focus forming assay.

2.5. Mouse model development and tissue tropism

All animal work was performed in accordance with UTMB policy as approved by the UTMB Institutional Animal Care and Use Committee (IACUC), protocol number 1807054, approved on 08/26/2018. Humane endpoints were utilized throughout to reduce animal pain and suffering, in accordance with the IACUC protocol.

To measure survival, male CD-1 mice (Strain 022, Charles River, Raleigh, NC), either four- or eight-weeks-old, were infected with $5.0 \log_{10}$ FFU (focus forming units) of either ILHV FSE 0800 or ILHV Original in a 100 µl volume either IP or SC in the back. Cohorts of ten (four-week old IP and eight-week-old SC) or five (eight-week-old SC) infected mice and five (four-week old IP and eight-week-old SC) or two (eight-week-old SC) mock-infected mice were observed out to 21 (eight-week-old IP), 24 (eight-week-old SC), or 28 days post-infection (four-week-old IP). Blood was collected retro-orbitally (RO) from half of the mice on days one and three post-infection and from the other half of the mice on days two and four post-infection. Serum was separated from the blood and stored at -80 °C. Mice were weighed daily through at least the first 14 DPI.

Survival was also assessed in six-to eight-week-old A129 mice (bred at UTMB, originally obtained from Marshall BioResources).

Mixed sex cohorts were injected SC with 5.0 log₁₀ FFU of either ILHV FSE 0800 (three female and four male) or ILHV Original (four female and three male), or mock-infected with PBS (three female and three male). Cohorts were weighed observed through three DPI, by which time ILHV was fully lethal in the infected mice.

To assess tissue tropism, four- and ten-week-old male CD-1 mice (Strain 022, Charles River, Raleigh, NC) were infected with 5.0 log₁₀ FFU of either ILHV FSE 0800 or ILHV Original in a 100 µl volume either IP or SC in the back. Cohorts of ten infected mice and four mock-infected mice were all bled RO on day one post-infection. On day two, half of the mice were euthanized for tissue harvest. The remaining mice were RO bled on additional time on day three, then euthanized on day four for tissue harvest. At the time of harvest, blood was collected from a terminal cardiac puncture and the animal was perfused with sterile PBS. Brain, liver, kidney, muscle (rear hamstring), heart, testes, lung, spleen, spinal cord, and eye tissue were harvested in MEM supplemented with 2% FBS and 1% PenStrep and stored at -80 °C, along with the serum that was separated from the blood. Tissues were thawed at the time of titration and homogenized for 1 min at a frequency of 26 sec⁻¹ in a TissueLyser (Qiagen, Haan, Germany) prior centrifugation for 5 min at 16,100×g to pellet debris. Focus forming assays were then performed as described above.

2.6. Vector competence

All mosquitoes (Table S3) were reared by the Insectary Services Core at UTMB under standard insectary conditions. Following bloodmeal feeds, all mosquitoes were held at 27 °C with 80% humidity and given access to 10% sucrose for the duration of the study.

Mosquito susceptibility to ILHV infection was determined via live feeds on viremic mice. Eleven- to twelve-week-old female A129 mice (bred at UTMB, originally obtained from Marshall BioResources) were infected SC in the back with 5.0 log₁₀ FFU of either ILHV FSE 0800 or ILHV Original. At two DPI, the mice were anesthetized with ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively) (for *Aedes* spp. mosquitoes) or ketamine/xylazine/acepromazine (100 mg/kg, 10 mg/kg, and 2 mg/kg, respectively) (for *Culex* spp. mosquitoes). A single mouse was used to feed each carton containing either 150 (for *Aedes* spp.) or 100 (for *Culex* spp.) female mosquitoes. The experiment was conducted using *Ae. aegypti* (Salvador (Brazil) and Venezuela), *Ae. albopictus* (Iquitos (Peru), and Salvador (Brazil)), *Cx. quinquefasciatus* (Salvador (Brazil) and Sebring), and *Cx. tarsalis* (CDC, Ft. Collins) mosquitoes (Table S3). The mosquitoes were allowed to feed for 15–30 min, after which time the mosquitoes were anesthetized by chilling and the visibly engorged mosquitoes were selected and returned to their cartons for the duration of the study. Mice were euthanized immediately following the mosquito feeds, and serum was collected for immediate titration via focus forming assay.

Mosquitoes were collected at both seven- and 14-days post-feed. At the time of harvest, mosquitoes were anesthetized by chilling and bodies and legs were collected and placed in homogenizer tubes with a homogenizer bead and 500 µl mosquito homogenization media (DMEM containing 2% FBS, 1% PenStrep, and 1% Fungizone (Gibco, Grand Island, NY)), then stored at -80 °C until ready for processing. For a subset of five mosquitoes per cohort in the *Aedes* spp. studies and 20 mosquitoes per cohort in the *Culex* spp. studies, saliva was also collected by immobilizing the mosquito with oil on a microscope slide, allowing the mosquito to salivate into a 10 µl pipette tip containing 8 µl FBS for 1 h, and ejecting the saliva/FBS mixture into 250 µl mosquito homogenization media to be stored at -80 °C until ready for processing.

The presence or absence of ILHV in mosquito bodies, legs, and saliva was determined via a cytopathic effect assay in Vero cells with immunostaining. Mosquito bodies and legs were homogenized for 1 min at a frequency of 26 sec⁻¹ in a TissueLyser. Samples were centrifuged for 5 min at 16,100×g to pellet debris. Finally, the media was removed from 96-well plates of Vero cells and replaced with 50 µl of Vero maintenance media supplemented with 1% Fungizone, and 100 µl of undiluted sample was added to the well. In addition to the experimental samples, each plate contained virus stocks as positive controls and homogenized, uninfected whole mosquitoes as negative controls. Following infection, the plates were kept in a 37 °C, 5% CO₂ humidified incubator and then fixed and stained as described for the focus forming assay. The presence of any detectable virus led the well in question to be considered positive. A subset of samples was later removed from the freezer for focus forming assays as previously described to quantitate the viral load.

2.7. Mosquito *OID*₅₀ determination

The *OID*₅₀ of ILHV FSE 0800 was determined in *Ae. aegypti* (Salvador) and *Ae. albopictus* (Salvador) mosquitoes. Artificial bloodmeals were generated by combining four parts serially diluted ILHV FSE 0800 in maintenance media with one part blood mixture consisting of 56% washed, packed human erythrocytes from whole blood (Gulf Coast Regional Blood Center, Houston, TX) supplemented with 20% FBS, 20% sucrose (10% w/v solution), and 4% ATP (100 mM solution). An aliquot of each bloodmeal was retained for titration simultaneous to feeding. The bloodmeals were presented to cartons of 100 female mosquitoes in warmed (37 °C) Hemotek feeders (Hemotek Ltd, Blackburn, UK) with mouse skins for 30–60 min. Mosquitoes were anesthetized by chilling and visibly engorged mosquitoes were then selected and maintained as previously described. Fourteen days after feeding on the bloodmeal, whole mosquitoes were anesthetized by chilling and collected in a homogenizer tube with 500 µl mosquito homogenization media and stored at -80 °C until ready for processing as previously described.

Following *OID*₅₀ determination, the susceptibility of a broad range of both *Ae. aegypti* and *Ae. albopictus* mosquito colonies to ILHV FSE 0800 was determined (Table S3). Bloodmeals were prepared as before, with the ILHV FSE 0800 concentration set to the previously calculated *OID*₅₀ level determined in *Ae. aegypti* (Salvador) and *Ae. albopictus* (Salvador). An aliquot was again retained for titration simultaneous to feeding, which took place as previously described. Fourteen days after feeding on the bloodmeal, mosquitoes were anesthetized by chilling and bodies were harvested. Samples were again stored at -80 °C until ready for processing as previously described above.

2.8. Statistical analysis

Survival differences for each cohort pair were assessed using the log-rank (Mantel-Cox) test, with the Holm-Sidak correction for multiple comparisons. Weight changes viral titers were analyzed by two-way repeated measures ANOVA with the strain and day as fixed factors and percent weight change or viral titer as the dependent variable. For replication kinetics data, and for murine data when all three cohorts (ILHV Original, ILHV FSE 0800, and mock) had at least one subject, differences between each cohort pair on a given day were assessed with Tukey's multiple comparison's test. When only two cohorts had at least one extant subject (4-week-old mice, IP route, days 9–14), cohorts were compared by multiple t-tests with the Holm-Sidak correction for multiple comparisons. OID_{50} values were calculated by probit analysis using SPSS v25 (IBM, Armonk, NY). Values below the limit of detection were treated as one-half of that limit for graphing and statistical purposes. For titers reported as FFU/g, for which the limit of detection is dependent on the exact weight of the collected tissue and is therefore variable from sample to sample, the lowest value for all samples of that tissue type was used as the limit of detection. All titer data was \log_{10} transformed prior to analysis to better approximate normality. An alpha of 0.05 was adopted as the cutoff for statistical significance for all tests a priori. Statistical analysis and graphing performed in Prism v10.0.0 (GraphPad, San Diego, CA). Raw data is available in Table S2, and the results of statistical analysis are available in Table S4.

3. Results

3.1. Viral replication in cultured cells

The replication kinetics of eight ILHV strains (Table S1) were characterized in seven cell lines. Four of the cell lines are mammalian: Vero (*Cercopithecus aethiops* kidney epithelial), Huh-7 (*Homo sapiens* hepatocyte), BHK (*Mesocricetus auratus* kidney fibroblast), and OK (*Didelphis marsupialis virginiana* kidney epithelial). Three of the cell lines are derived from the larva of *Ae. albopictus* mosquitoes: C6/36, C7/10, and U4.4. All eight ILHV strains exhibited robust replication in all seven cell lines (Fig. 1). In mammalian cells (Fig. 1A–D), ILHV rapidly reached its peak titer of $7.14\text{--}7.83 \log_{10}$ FFU/ml, most often at three days post-infection (DPI) (range: 2–4 DPI). The post-peak decline in ILHV titers corresponded with the onset of cytopathic effect (CPE) in the cells (Figs. S1A–D). In contrast, ILHV

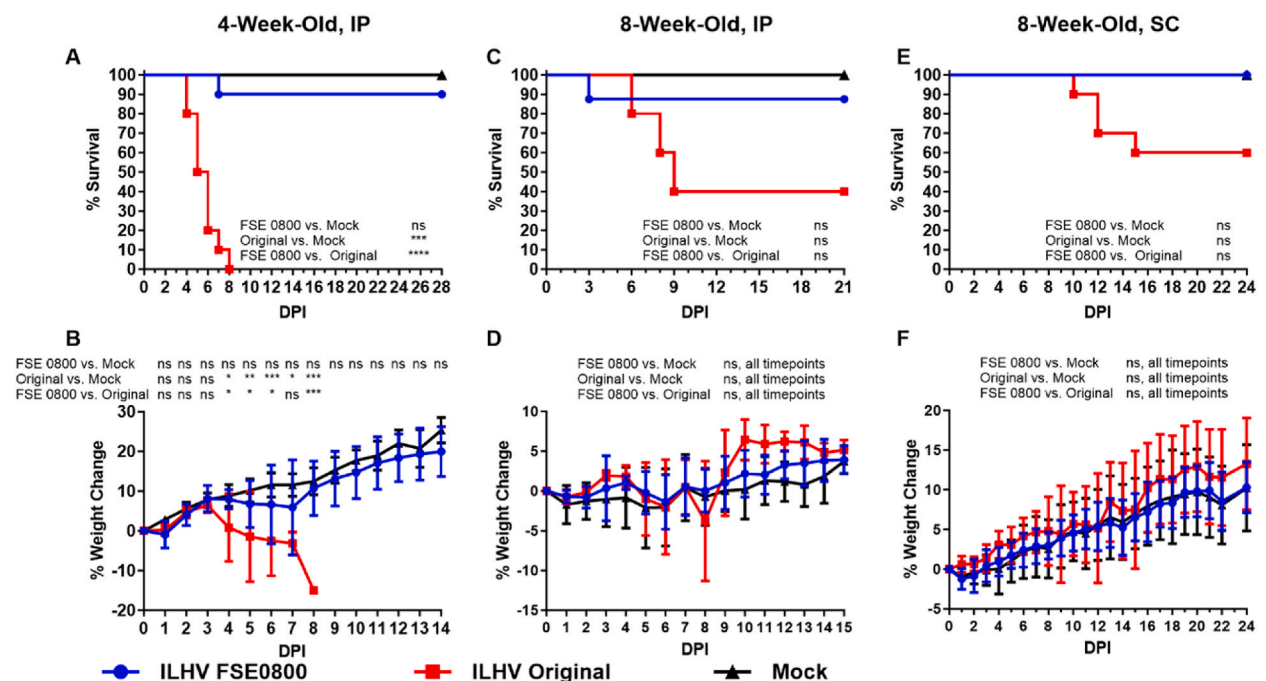


Fig. 2. Mortality and weight loss in a CD-1 model is highly dependent on ILHV strain and mouse age. (A–B) four- or (C–F) eight-week-old male CD-1 mice were infected via either the (A–D) intraperitoneal or (E–F) subcutaneous route with $5.0 \log_{10}$ FFU of ILHV FSE 0800, ILHV Original, or a PBS-only mock control. (A, C, E) Survival rates and (B, D, F) weight change are reported. Colors represent the ILHV strain or mock cohort. For weight loss, symbols represent the mean and error bars represent the standard deviation. The (A–B) four-week-old IP route and (E–F) eight-week-old SC route was assessed in $n = 10$ ILHV FSE 0800- and ILHV Original-infected mice and in $n = 5$ mock-infected mice. The (C–D) eight-week-old IP route was assessed in $n = 5$ ILHV FSE 0800- and ILHV Original-infected mice and in $n = 2$ mock-infected mice. Data are representative of a single experiment. Survival was assessed by the log-rank (Mantel-Cox) test, with the Holm-Sidak correction for multiple comparisons. Weight changes were assessed by two-way ANOVA with Tukey's correction for multiple comparisons, except for days 9–14 in the four-week-old model (B) which required multiple t-tests with the Holm-Sidak correction due to the presence of two rather than three cohorts. ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

replication in the three *Ae. albopictus* cell lines was characterized by delayed peak titers without an appreciable post-peak decline (Fig. 1E–G). This is consistent with visual observations of the infected mosquito cell monolayers, which exhibited clumping and syncytia formation but not the destructive CPE seen in the mammalian cell lines (Figs. S1E–G). The two RNAi-deficient *Ae. albopictus* cell lines, C6/36 and C7/10, supported peak ILHV titers of 6.81–8.06 \log_{10} FFU/ml and 8.20–9.54 \log_{10} FFU/ml, respectively. In contrast, the RNAi-competent U.4.4 line only supported peak ILHV titers of 5.73–6.45 \log_{10} FFU/ml. Strain based variation in ILHV replication was generally non-significant or weakly significant, especially at or prior to peak titer. Significant differences were more common at later timepoints, when degraded or destroyed cell monolayers are more likely to have a prominent impact (Fig. S1 and Table S4). These results support the serologic and mosquito survey evidence for the ability of ILHV to productively infect a broad range of potential host and vector species.

3.2. Establishment of murine models of pathogenesis

There is no well-characterized small animal model for ILHV infection and disease. Therefore, four-week-old male CD-1 mice were infected with 5.0 \log_{10} FFU of either ILHV FSE 0800 or ILHV Original, or mock-infected with PBS, via the intraperitoneal (IP) route (Fig. 2A and B). The FSE 0800 and Original strains of ILHV represent the temporal extremes of our available ILHV panel (Table S1); FSE 0800 was isolated in 2004 and has been passaged four times in Vero cells and is considered as close to non-adapted, whereas Original was isolated in 1944 and has been passaged 29 times in suckling mice and twice in Vero cells, and thus is considered mouse-adapted. ILHV Original was universally lethal, with an average survival time of 5.6 days and all mice succumbing to infection by eight DPI. ILHV FSE 0800, in contrast, was only lethal in 10% of mice. Mice that succumbed to ILHV infection exhibited hunched posture (8/11), ruffled fur (6/11), lethargy (6/11), tremors (5/11), and grimace (3/11), with onset either the day of death or one day prior. ILHV Original-infected mice lost a significant amount of weight compared to mock-infected mice on days four through eight post-infection, corresponding to the timeframe during which ILHV Original-infected mice died. The ILHV Original-infected mice also lost significantly

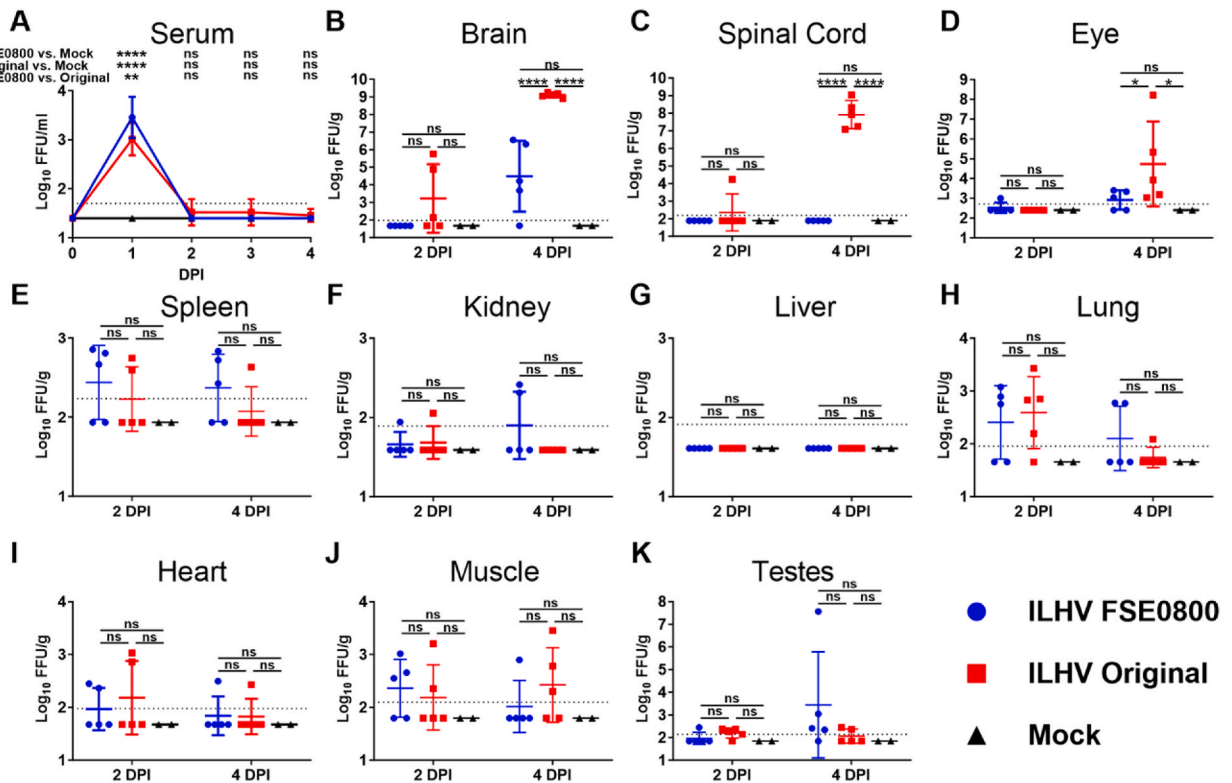


Fig. 3. ILHV replicates in multiple murine tissues. Four-week-old male CD-1 mice were infected via the intraperitoneal route with 5.0 \log_{10} FFU of ILHV FSE 0800 ($n = 10$), ILHV Original ($n = 10$), or a PBS-only mock control ($n = 4$). Blood was collected from all extant subjects via the retro-orbital route at 1 DPI and 3 DPI. Half of the subjects from each cohort were euthanized at 2 DPI, and the remaining half were euthanized at 4 DPI. At the time of euthanasia, blood was collected prior to PBS perfusion, after which solid organs were collected. Infectious ILHV was detected by focus forming assay in Vero cells. Data are representative of a single experiment. For (A) serum samples, symbols represent the mean and error bars represent the standard deviation. For (B–K) solid tissues, symbols represent individual subjects, midline bars represent the mean, and error bars represent the standard deviation. Dotted lines represent the lower limit of detection. Samples with no detectable ILHV are represented as half the lower limit of detection. \log_{10} -transformed titers were analyzed by two-way ANOVA with Tukey's multiple comparison's test. ns = $p \geq 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Table 1

ILHV is poorly disseminated in *Culex* spp. mosquitoes. *Culex* spp. mosquitoes were permitted to feed upon A129 mice previously infected with either ILHV FSE 0800 or ILHV Original. Engorged mosquitoes were maintained for an EIP of seven or 14 days. Bodies, legs, and saliva were collected and the presence of ILHV was detected by infectious assay with immunostaining in Vero cells. ILHV titers detected in the sera of A129 mice immediately following mosquito feeding are reported in FFU/ml. Infection is defined as ILHV in the body, dissemination is defined as ILHV in the legs, and transmission is defined as ILHV in the saliva. Data are representative of a single experiment.

| Mosquito | | ILHV | ILHV | EIP | Infection | Dissemination | | Transmission | | |
|-------------------------------|--------------|----------|-------------|-----------------|---|---|---|---|---|---|
| Species | Colony | Strain | Titer | | % ($\frac{\# \text{ Infected}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Disseminated}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Disseminated}}{\# \text{ Infected}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Infected}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Disseminated}}$) |
| <i>Culex quinquefasciatus</i> | Sebring | FSE 0800 | 7.93 | 7 | 0 (0/53) | 0 (0/53) | N/A (0/0) | 0 (0/20) | N/A (0/0) | N/A (0/0) |
| | | | 8.13 | 14 | 1.5 (1/68) | 1.5 (1/68) | 100 (1/1) | 5.0 (1/20) | 100 (1/1) | 100 (1/1) |
| | | Original | 8.49 | 14 | 4.0 (3/75) | 0 (0/20) | N/A (0/0) | 0 (0/20) | N/A (0/0) | N/A (0/0) |
| | | | 8.43 | 7 | 8.8 (5/57) | 0 (0/57) | 0 (0/5) | 0 (0/20) | 0 (0/4) | N/A (0/0) |
| | Salvador | FSE 0800 | 8.72 | 14 | 0 (0/72) | 0 (0/72) | N/A (0/0) | 0 (0/20) | N/A (0/0) | N/A (0/0) |
| | | | 8.18 | 14 | 2.8 (2/71) | 0 (0/20) | N/A (0/0) | 0 (0/20) | N/A (0/0) | N/A (0/0) |
| | | Original | 7.78 | 14 | 35.7 (10/28) | 5.0 (1/20) | 14.3 (1/7) | 5.0 (1/20) | 14.3 (1/7) | 100 (1/1) |
| | | | 8.28 | 14 | 5.0 (1/20) | 0 (0/20) | N/A (0/0) | 0 (0/20) | N/A (0/0) | N/A (0/0) |
| <i>Culex tarsalis</i> | Fort Collins | FSE 0800 | 8.04 & 8.34 | 14 ^a | 94.1 (16/17) | 70.6 (12/17) | 68.8 (11/16) | 0 (0/17) | 0 (0/16) | 0 (0/12) |
| | | Original | 8.28 & 8.32 | 14 ^b | 68.8 (11/16) | 50.0 (8/16) | 54.5 (6/11) | 0 (0/16) | 0 (0/11) | 0 (0/8) |

^a One mosquito in the cohort had a negative body and positive legs.

^b Two mosquitoes in the cohort had negative bodies and positive legs.

Table 2

ILHV is efficiently disseminated in *Aedes* spp. mosquitoes. *Aedes* spp. mosquitoes were permitted to feed upon A129 mice previously infected with either ILHV FSE 0800 or ILHV Original. Engorged mosquitoes were maintained for an EIP of seven or 14 days. Bodies, legs, and saliva were collected and the presence of ILHV was detected by infectious assay with immunostaining in Vero cells. ILHV titers detected in the sera of A129 mice immediately following mosquito feeding are reported in FFU/ml. Infection is defined as ILHV in the body, dissemination is defined as ILHV in the legs, and transmission is defined as ILHV in the saliva. Data are representative of a single experiment.

| Mosquito | | ILHV Strain | ILHV Titer | EIP | Infection | Dissemination | | Transmission | | | |
|-------------------------|----------------|-------------|------------|-----|---|---|---|---|---|---|-----------|
| Species | Colony | | | | % ($\frac{\# \text{ Infected}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Disseminated}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Disseminated}}{\# \text{ Infected}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Engorged}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Infected}}$) | % ($\frac{\# \text{ Transmitting}}{\# \text{ Disseminated}}$) | |
| <i>Aedes aegypti</i> | Iquitos | FSE 0800 | 8.34 | 7 | 100 (36/36) | 100 (36/36) | 100 (36/36) | 100 (5/5) | 100 (5/5) | 100 (5/5) | |
| | | Original | 8.34 | 7 | 100 (25/25) | 100 (25/25) | 100 (25/25) | 80 (4/5) | 80 (4/5) | 80 (4/5) | |
| | Salvador (F10) | FSE 0800 | 8.18 | 7 | 14 ^a | 96.4 (27/28) | 100 (28/28) | 100 (27/27) | 40 (2/5) | 40 (2/5) | 40 (2/5) |
| | | Original | 8.40 | 7 | 14 ^a | 91.5 (43/47) | 89.4 (42/47) | 97.7 (42/43) | 40 (2/5) | 40 (2/5) | 40 (2/5) |
| | | FSE 0800 | 8.40 | 7 | 14 ^b | 98.3 (57/58) | 100 (58/58) | 100 (57/57) | 80 (4/5) | 80 (4/5) | 80 (4/5) |
| | | Original | 8.40 | 7 | 14 ^b | 78.7 (37/47) | 38.3 (18/47) | 43.2 (16/37) | 60 (3/5) | 75 (3/4) | 75 (3/4) |
| <i>Aedes albopictus</i> | Salvador (F10) | FSE 0800 | 8.40 | 14 | 91.2 (52/57) | 71.9 (41/57) | 75.0 (39/52) | 20 (1/5) | 20 (1/5) | 25 (1/4) | |
| | | Original | 8.48 | 7 | 100 (19/19) | 94.7 (18/19) | 94.7 (18/19) | 100 (5/5) | 100 (5/5) | 100 (5/5) | |
| | Venezuela | FSE 0800 | 8.51 | 7 | 14 | 100 (25/25) | 44.0 (11/25) | 44.0 (11/25) | 0 (0/5) | 0 (0/5) | 0 (0/1) |
| | | Original | 8.18 | 7 | 14 | 94.6 (35/37) | 70.3 (26/37) | 74.3 (26/35) | 20 (1/5) | 25 (1/4) | 100 (1/1) |
| | | FSE 0800 | 8.51 | 7 | 14 | 100 (37/37) | 100 (37/37) | 100 (37/37) | 0 (0/5) | 0 (0/5) | 0 (0/5) |
| | | Original | 8.18 | 7 | 14 | 100 (33/33) | 100 (33/33) | 100 (33/33) | 80 (4/5) | 80 (4/5) | 80 (4/5) |
| | | | | 14 | 100 (29/29) | 55.2 (16/29) | 55.2 (16/29) | 0 (0/5) | 0 (0/5) | 0 (0/3) | |
| | | | | 14 | 100 (40/40) | 100 (40/40) | 100 (40/40) | 40 (2/5) | 40 (2/5) | 40 (2/5) | |

^a One mosquito in the cohort had a negative body and positive legs.

^b Two mosquitoes in the cohort had negative bodies and positive legs.

more weight than the ILHV FSE 0800-infected mice during this time except for day seven, the day on which a single ILHV FSE 0800 mouse succumbed to infection.

Eight-week-old male CD-1 mice were also infected with $5.0 \log_{10}$ FFU of either ILHV FSE 0800 or ILHV Original, or mock-infected with PBS, via either the IP route (Fig. 2C and D) or the subcutaneous (SC) route (Fig. 2E and F) to assess the potential of the CD-1 mouse model in future studies requiring an older mouse, such as the challenge phase of a vaccine study. ILHV Original was again more virulent than ILHV FSE 0800 in both models, although this difference did not meet the threshold of significance with the cohort sizes utilized. Whereas ILHV Original was 100% lethal in four-week-old IP-infected mice, it was only 60% lethal in eight-week-old IP-infected mice and 40% lethal in eight-week-old SC-infected mice. Time to death was longer in the older models, with ILHV Original resulting in an average survival time of 7.7 days following IP injection and 12.3 days following SC injection. Neither strain of ILHV resulted in significant weight loss in the eight-week-old model. Visible signs of illness were similar to those observed in the four-week-old CD-1 mouse model. In addition to the eight-week-old CD-1 mouse model, six- to eight-week-old A129 mice, of both sexes, were injected SC with $5.0 \log_{10}$ FFU of either ILHV FSE 0800 or ILHV Original, or mock-infected with PBS (Fig. S2). This *Ifnar^{-/-}* model was rapidly and uniformly lethal following ILHV infection with either strain, with 100% of mice infected with either ILHV strain succumbing on day three post-infection.

Tropism was assessed in the four-week-old CD-1 model at two and four DPI (Fig. 3). Viremia was modest in magnitude and duration (Fig. 3A). At one DPI, ILHV FSE 0800 and ILHV Original reached mean titers of $3.5 \log_{10}$ FFU/ml and $3.0 \log_{10}$ FFU/ml, respectively, with all subjects above the limit of detection. Viremia for both strains was below the limit of detection at days two through four post-infection with the exception of a single ILHV Original mouse from the day two and the day three cohorts being viremic at the limit of detection on those days. ILHV Original was highly neurotropic, reaching $3.2 \log_{10}$ FFU/g in the brain at two DPI and $9.1 \log_{10}$ FFU/g at four DPI (Fig. 3B). ILHV FSE 0800, on the other hand, was not detected in the brain on day two and reached only $4.5 \log_{10}$ FFU/g on day four. Similar results were observed in the spinal cord and the eye (Fig. 3C and D). Virus was detected at low levels (2.4 – $2.9 \log_{10}$ FFU/g) in the spleen of 60% of ILHV FSE 0800-infected mice at days two and four post-infection, and at 60% and 20% of ILHV Original-infected mice at days two and four post-infection, respectively (Fig. 3E). Viral load in the kidneys was similarly low in magnitude and prevalence (Fig. 3F). No virus was detected in the liver (Fig. 3G). The virus was present at low levels in lung and heart tissue following both ILHV FSE 0800 and ILHV Original infection, with the magnitude and prevalence decreasing from day two to day four in both tissues for each strain (Fig. 3H and I). Similar titers were detected in somatic muscle tissue, although there was no trend toward decreasing titer from day two to day four (Fig. 3J). Infectious virus was also detected in the testes (Fig. 3K). ILHV Original generated relatively low titers (2.1 – $2.4 \log_{10}$ FFU/g) in 80% of mice on day two and in 40% of mice on day four. ILHV FSE 0800 was detected in 20% of the mice at day two and 80% of the mice at day four. The majority of ILHV FSE 0800-positive mice had similar viral loads (2.3 – $3.0 \log_{10}$ FFU/g) compared to the ILHV Original-positive mice, but one ILHV FSE 0800 mouse from day four had $7.6 \log_{10}$ FFU/g in its testes. A parallel tropism study was conducted on older (ten-week-old) CD-1 mice (Fig. S3). Viremia once again peaked at one DPI and essentially disappeared by two DPI. However, viral loads in the solid organs were generally decreased both in magnitude and in the percentage of virus-positive subjects compared to the four-week-old mice.

3.3. Vector competence

To establish the vector competence of ILHV, *Culex* spp. and *Aedes* spp. mosquitoes were allowed to feed on viremic A129 mice infected with either ILHV FSE 0800 or ILHV Original. Engorged mosquitoes were maintained for an extrinsic incubation period (EIP) of seven or 14 days, at which times bodies, legs, and saliva were harvested and the presence or absence of ILHV was assessed by cytopathic effect assay in Vero cells with immunostaining. The Sebring colony of *Cx. quinquefasciatus* was highly refractory to both strains of ILHV (Table 1). The Salvador colony of *Cx. quinquefasciatus* was also generally poorly infected by ILHV; while the FSE 0800 strain infected 35.7% of the bodies, only a single mosquito had a disseminated and transmissible infection. The *Cx. tarsalis* mosquitoes were more susceptible than the *Cx. quinquefasciatus* mosquitoes. ILHV FSE 0800 was more infectious than ILHV Original in *Cx. tarsalis*, but once again the virus was poorly disseminated; of the 94.1% of *Cx. tarsalis* with ILHV FSE 0800-infected bodies, only 68.8% developed a disseminated infection as assessed by virus in the legs and no mosquitoes became able to transmit the virus as assessed by the absence of virus in the saliva.

In contrast, *Aedes* spp. mosquitoes were highly susceptible to infection by ILHV (Table 2). ILHV FSE 0800 was generally more infectious than ILHV Original in the *Aedes* spp. mosquitoes, like the pattern seen in the more refractory *Culex* mosquitoes. In *Ae. aegypti*, ILHV FSE 0800 infected over 90% of bodies following an EIP of seven or 14 days in both the Iquitos and Salvador colonies. ILHV Original infected 79–81% of bodies after seven days and 91–96% of bodies after 14 days. ILHV FSE 0800 disseminated to the legs of infected *Ae. aegypti* quickly and at a high rate, with 98–100% of infected mosquitoes developing a disseminated infection by day seven. ILHV Original disseminated in only 33–43% of infected mosquitoes by day seven but reached 75–100% dissemination by day 14. In further contrast to the infection of *Cx.* mosquitoes, *Ae. aegypti* had virus present in the saliva of 40–100% of FSE 0800-infected mosquitoes and 25–75% of Original-infected mosquitoes with disseminated infections. The results from *Ae. albopictus* were broadly like those of *Ae. aegypti*. ILHV FSE 0800 infected and disseminated to 95–100% of mosquitoes and was present in the saliva of 80–100% of *Ae. albopictus* mosquitoes by day 14. ILHV Original similarly infected 95–100% of bodies but lagged somewhat in dissemination to the legs, infecting only 44–55% of legs on day seven but reaching 70–100% of legs on day 14. Interestingly, although both *Ae. aegypti* and *Ae. albopictus* had high rates of ILHV in the bodies and legs by day seven, only *Ae. aegypti* had ILHV in the saliva by day seven, indicating a more rapid onset of transmission competence in *Ae. aegypti* than in *Ae. albopictus*. This indication that ILHV likely utilizes *Aedes* spp. rather than *Culex* spp. as its primary vector was broadly reflected in the titers of ILHV detected in the bodies, legs, and saliva of *Ae. aegypti*, *Ae. albopictus*, and *Cx. tarsalis* mosquitoes (Fig. S4).

As *Aedes* spp. of mosquitoes were competent vectors for ILHV following a live feed on a highly viremic (8.18–8.51 log₁₀ FFU/ml) mice, artificial bloodmeals were utilized to feed mosquitoes with progressively lower concentrations of virus to determine the oral infectious dose 50% (OID₅₀). ILHV FSE 0800 was selected for OID₅₀ determination due to its generally higher infectivity in mosquitoes and lower passage history. The OID₅₀ in *Ae. aegypti* Salvador was 6.5 log₁₀ FFU/ml, and the OID₅₀ for *Ae. albopictus* Salvador was 5.5 log₁₀ FFU/ml (Fig. 4A). To determine whether the susceptibility of the Salvador colonies was broadly representative of *Ae. aegypti* and *Ae. albopictus* or a colony-specific artifact, a geographically diverse panel of *Ae. aegypti* and *Ae. albopictus* were fed artificial bloodmeals with ILHV FSE 0800 at the calculated OID₅₀ concentrations (Fig. 4B and C). Both species were broadly susceptible across all colonies tested. The eight colonies of *Ae. aegypti* averaged 67% infection (range: 28–100%); the only colony with an infection rate below 50% was Dakar, which was also the lone representative from Africa. The six colonies of *Ae. albopictus* averaged 65% infection (range: 52–74%).

4. Discussion

Although ILHV was discovered in 1944, relatively little is known about this virus outside of serosurveys and case reports from sporadic symptomatic human infections [1,8,11,13,26–30,33,34,36,37,40,41,44,48–53]. Given that related flaviviruses such as Bussuquara virus, Cacipacoré virus, dengue virus (DENV), Zika virus (ZIKV), WNV, Saint Louis encephalitis virus (SLEV), yellow fever virus (YFV), and ROCV are endemic throughout the reported geographic range of ILHV [26,30,54,55], and the tendency for serological cross reactivity between flaviviruses [56–59], the actual disease burden of ILHV is difficult to assess and quantify. Nonetheless, to date, no large-scale human epidemics or epizootic amplifications have been reported. In the past three decades, both WNV and ZIKV have been introduced to the New World and rapidly became endemic [60–63]. The spread and endemicity of WNV in the Americas underscore the potential for these viruses to rapidly become a serious public health concern out of relative obscurity. Against the landscape of factors such as increasing urbanization of natural biomes, forest degradation for agricultural use, and climate change, the incidence of humans encountering wild animals is ever increasing, thereby increasing the potential for zoonotic spillover events [46, 64,65]. Proactively developing a solid knowledge foundation as well as agent-specific reagents and models for under-characterized zoonoses with a strong potential for emergence will allow public health and research efforts to more efficiently respond to future outbreaks.

ILHV has been isolated from humans as well as from a variety of bird and mosquito species [1,5,6,8–17]. However, serologic evidence indicates that a wide range of vertebrates may be susceptible hosts, including additional bird species as well as mammals and reptiles [7,16,24–38]. Such a range would not be unprecedented; WNV has been detected in nearly 300 bird species and over 30 other species including mammals, reptiles, and amphibians [66,67]. Combined with the diverse habitats and feeding behaviors of the mosquito species in which the virus has been detected, ILHV may exist in several different transmission cycles. As this knowledge is necessary to efficiently deploy surveillance and preventative measures such as mosquito spraying, we endeavored to characterize ILHV replication in cell lines from a variety of species. We found that all seven cell lines supported robust replication of all eight ILHV strains tested. Increased peak titers in the RNAi-deficient *Ae. albopictus* cell lines (C6/36 and C7/10) compared to the RNAi-intact *Ae. albopictus* cell line (U4.4) suggests that ILHV is indeed sensitive to the RNAi response of mosquitoes; similar RNAi sensitivities have been reported for other flaviviruses including DENV and YFV [68,69]. Peak titers were remarkably similar across the vertebrate cell lines

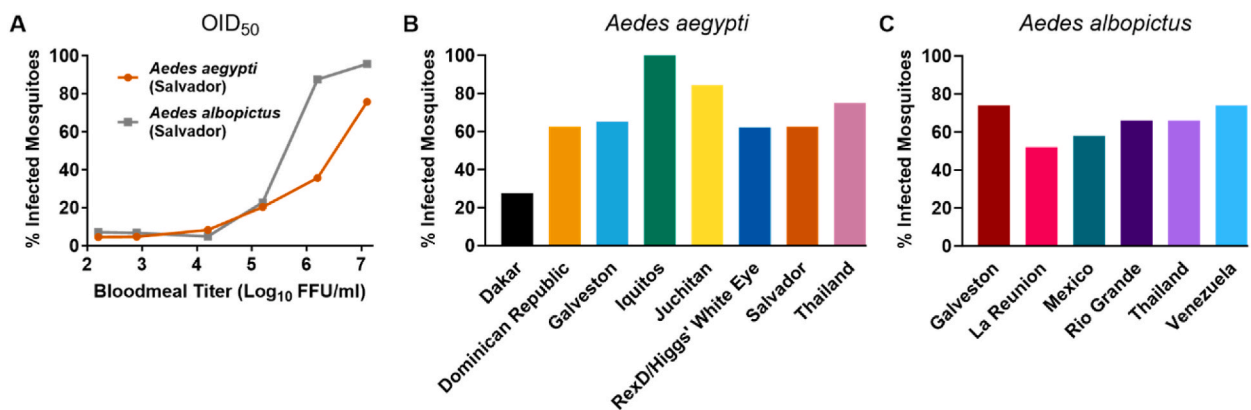


Fig. 4. ILHV efficiently infects *Aedes* spp. mosquitoes from a broad geographic range. To determine the OID₅₀ of ILHV in *Aedes* spp. mosquitoes, (A) *Ae. aegypti* and *Ae. albopictus*, both originating from Salvador, Brazil, were fed artificial bloodmeals containing 2.2–7.1 log₁₀ FFU/ml of ILHV FSE 0800. To determine the susceptibility of (B) *Ae. aegypti* and (C) *Ae. albopictus* from a broad geographic range to ILHV infection, mosquitoes from various colonies were fed artificial bloodmeals containing ILHV FSE 0800 at the OID₅₀ concentration calculated for *Ae. aegypti* (Salvador) and *Ae. albopictus* (Salvador), respectively. In all experiments, engorged mosquitoes were maintained for an EIP of 14 days prior to harvest and the presence or absence of ILHV was determined by infectious assay with immunostaining in Vero cells. OID₅₀ values (A) were calculated by probit analysis using the rates of infection calculated from an average of 38 (range: 22–59) *Ae. aegypti* and 40 (range: 23–57) *Ae. albopictus* per dose. Rates of infection from geographically diverse *Aedes* spp. mosquitoes (B–C) were calculated from an average of 28 (range: 8–40) *Ae. aegypti* and from 50 *Ae. albopictus* per colony. Data are representative of a single experiment.

despite representing primates, rodents, and marsupials. This suggests that the wide-ranging detection of antibodies against ILHV may be due to productive ILHV infections rather than cross-reactivity following infection with a related flavivirus. Concerted efforts to isolate ILHV or detect ILHV genomes in samples from a variety of species as part of surveillance efforts will be required to confirm this finding and to better define the transmission cycle(s) of ILHV.

Reproducible animal models are necessary to investigate the pathogenesis of emerging agents as well as to evaluate candidate therapeutics and vaccines. We aimed to develop a series of models of ILHV infection that: (1) recapitulates human disease; (2) does not require adapted ILHV strains, which may preclude studies with more relevant, circulating ILHV strains; (3) does not require transgenic animals with human gene orthologs, which are frequently expensive and may not be readily available to researchers in all regions; (4) does not require genetic or transient immunosuppression, to facilitate vaccine studies and investigations of virus-host interactions which depend upon an intact immune response; and finally (5) produces readily measured phenotypes such as lethality, morbidity, or viremia to evaluate the efficacy of medical countermeasures. We found that CD-1 mice are a desirable model for ILHV infection, and that this model exhibits ILHV strain-specific phenotypes. Infection of young (four-week-old) mice leads to substantial weight loss and uniform lethality following ILHV Original infection, making this older, more highly passaged strain ideal for testing candidate therapeutics and vaccines. Tropism studies revealed high titers of ILHV Original in the brain and spinal cord; this is consistent with the reported neurological symptoms in severe human infections [11,13,44]. The FSE 0800 strain was also neuroinvasive, albeit to a lesser degree than the Original strain, but did not induce significant weight loss or lethality. These divergent strain-based phenotypes may indicate this model's utility in detecting emergent phenotypes should new mutations or genotypes arise within ILHV. Interestingly, 80% of mice had detectable ILHV FSE 0800 titers in the testes at four DPI, with one measured at $7.6 \log_{10}$ FFU/g. This tropism merits further investigation, particularly considering the sexual transmission and testicular damage caused by the related flavivirus ZIKV [70–73]. Older (eight-week-old) CD-1 mice succumbed to infection at a rate of 60% following IP inoculation with ILHV Original; this provides a useful model for vaccine efficacy studies, which necessitate the use of older animals to accommodate seroconversion following vaccination. Finally, we characterized ILHV in A129 mice, which are *Ifnar*^{-/-}. This model was rapidly and uniformly lethal and consistently developed high levels of viremia. Although immunocompromised, this does provide an extremely stringent model for medical countermeasure testing as well as an important tool for vector competence studies.

It has long been assumed that ILHV is primarily maintained in a transmission cycle between birds and *Culex* spp. mosquitoes. Phylogenetic analyses consistently demonstrate that ILHV is closely related to other flaviviruses such as JEV, SLEV, and WNV that can utilize bird species as amplifying hosts, *Culex* spp. mosquitoes as vectors, and can cause encephalitic disease during spillover infections in humans [46,74–77]. Direct evidence supporting a *Culex* spp. driven transmission cycle for ILHV includes the detection of ILHV RNA in *Cx. (Melanoconion) spp.*, *Cx. declarator*, and *Cx. portesi* and the isolation of ILHV from *Cx. coronator* and diverse species of birds: the little blue heron (*Egretta caerulea*), the shiny cowbird (*Molothrus bonariensis*), the keel-billed toucan (*Rhamphastos sulfuratus*), and the double-collared seedeater (*Sporophila caerulea*) [4,5,7,16,17]. However, we found that two colonies of *Cx. quinquefasciatus*, an important vector for WNV and SLEV which is found in ILHV-endemic regions [78], were largely refractory to disseminated infection by both ILHV Original and ILHV FSE 0800 despite engorging on high titer (7.78 – $8.49 \log_{10}$ FFU/ml) bloodmeals from viremic mice. The highest rate of infection we detected in the *Cx. quinquefasciatus* Sebring colony was 9%; in contrast, WNV has been reported to infect this same mosquito colony at rates of 91–100% when exposed to comparable titer bloodmeals [79]. *Cx. tarsalis* was much more susceptible to infection and dissemination, but ILHV was not detected in any saliva samples and *Cx. tarsalis*' geographic range does not overlap with ILHV-endemic areas, raising questions as to its vector potential. Interestingly, *Ae. aegypti* and *Ae. albopictus* were far more susceptible to infection and dissemination to both the legs and saliva. Dissemination to the legs, which has been proposed as a more sensitive measure of true transmission potential than forced salivation [80], was especially pervasive and rapid with the more modern, lower passage FSE 0800 strain of ILHV. Infection and dissemination to the legs by ILHV FSE 0800 was nearly universal by day seven in both colonies of *Ae. aegypti* and *Ae. albopictus*; by day 14, dissemination to the saliva was detected in at least 80% of mosquitoes in all colonies. The high rates of infection in *Aedes* spp. were widespread amongst colonies from different regions of North America, South America, and Asia; the only colony with modest rates of infection was an *Ae. aegypti* colony from Dakar, Senegal. Additional mosquito colonies from Africa must be tested to determine whether this is a coincidence or an accurate reflection of a New World flavivirus that infects African mosquitoes less efficiently. Vector competence studies are difficult to directly compare across studies due to the potential impacts of the source, generation, rearing of the mosquitoes, the specific composition and source of the bloodmeal, and the viral stock preparation and detection methods. However, our calculated OID_{50} values of $5.5 \log_{10}$ FFU/ml in *Ae. albopictus* and $6.5 \log_{10}$ FFU/ml in *Ae. aegypti* are broadly in line with the approximately 5 – $6 \log_{10}$ FFU/ml range most commonly reported for DENV and YFV, both of which are demonstrated to be efficiently transmitted to humans by *Aedes* spp. mosquitoes [81–85]. Taken in whole, this work raises the possibility that *Aedes* spp. mosquitoes may play an important role now, or possibly in the future, in ILHV transmission despite previous assumptions that *Culex* spp. would be the primary ILHV vectors.

This study has several limitations. All virus quantification was conducted by focus forming assay to detect live, replication-competent viral particles; additional studies utilizing genome-based quantification methods may yield additional insight into viral replication and tropism. The most modern ILHV strain utilized in this study was isolated in 2004. While ILHV has been detected more recently, these studies have utilized PCR and virus isolation was not attempted. Efforts toward viral isolation as a parallel detection method would likely yield more recent ILHV strains which would aid in monitoring for phenotypic and sequence changes. Additionally, while *in vitro* replication kinetics included a wide panel of ILHV, *in vivo* characterization was performed only for the oldest (Original) and newest (FSE 0800) strains in the panel; future studies with additional ILHV strains may reveal important differences, particularly between the more modern, lower passage isolates. Cultured cells are imperfect models of viral replication; notably, Vero cells are deficient in the synthesis of interferon (IFN) and C6/36 and C7/10 cells are deficient in RNA interference (RNAi) [86–89]. However, they remain an important method of evaluating viral replication in a wide variety of species, many of which are not suitable

or common laboratory models but may be important to the viral transmission cycle. We utilized cell lines to investigate the range of species that can support ILHV replication; however, our panel was limited and did not include cells derived from neurologic tissue, birds, or non-*Ae. albopictus* mosquito species. While not all species have cell lines available, including additional diverse lines or incorporating primary cells would be desirable in future experiments. Furthermore, replication competence in cell culture does not necessarily indicate replication competence in the species from which those cells were derived. Confirming any species as susceptible to ILHV will require detection of genomic material or, ideally, viral isolation from that species. Our animal model development focused on viral load, morbidity, and mortality; future investigations should directly examine pathology and immune markers. We focused on male mice for model development to examine potential parallels between ILHV and findings of testicular tropism and damage following ZIKV infection; future studies should include female mice to examine any potential sex-based differences in viral replication or pathology. Finally, our vector competence studies focused on *Aedes* spp. and *Culex* spp. mosquitoes, as these are the main vectors for the most well characterized flaviviruses. However, it is possible that ILHV is primarily vectored by a different genus. In particular, ILHV has been isolated multiple times from *Psorophora ferox*, and this species was implicated in the 1975–1976 outbreak of ROCV [1, 39,90]. ILHV has also been isolated from *Ae. scapularis* and *Sabethes chloropterus*, important bridge and sylvan vectors, respectively [8, 9,91,92]. Expanding the range of species utilized in vector competence studies would provide a more comprehensive picture of ILHV transmission cycles, and thus shed more light on its potential for emergence.

Ethics statement

All animal work was performed in accordance with University of Texas Medical Branch (UTMB) policy as approved by the UTMB Institutional Animal Care and Use Committee (IACUC), protocol number 1807054, approved on 08/26/2018. Humane endpoints were utilized throughout to reduce animal pain and suffering, in accordance with the IACUC protocol.

Data availability statement

All raw data described in this paper (Table S2) and the results of the statistical analysis (Table S4) are available for access through Mendeley Data - <https://doi.org/10.17632/4yn4jnw5h.1>.

This paper does not report original code. Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request.

Materials availability

Further information and requests for resources and reagents should be directed to the lead contact, Nikos Vasilakis (nivasila@utmb.edu). All ILHV strains are available from the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA) at the University of Texas Medical Branch under a material transfer agreement (MTA). Contact Ken Plante (ksplante@utmb.edu).

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CRediT authorship contribution statement

Kenneth S. Plante: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jessica A. Plante:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Sasha R. Azar:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Divya P. Shinde:** Writing – review & editing, Investigation. **Dionna Scharton:** Writing – review & editing, Investigation. **Alice F. Versiani:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Natalia Ingrid Oliveira da Silva:** Writing – review & editing, Investigation. **Taylor Strange:** Writing – review & editing, Investigation. **Lívia Sacchetto:** Writing – review & editing, Investigation. **Eric B. Fokam:** Writing – review & editing, Investigation. **Shannan L. Rossi:** Writing – review & editing, Investigation. **Scott C. Weaver:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Rafael E. Marques:** Writing – review & editing, Resources. **Mauricio L. Nogueira:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Nikos Vasilakis:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Nikos Vasilakis reports financial support was provided by National Institutes of Health. Scott C. Weaver reports financial support was provided by National Institutes of Health. Mauricio L. Nogueira reports financial support was provided by State of Sao Paulo Research Foundation. Mauricio L. Nogueira and Rafael Elias Marques reports financial support was provided by CNPq. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

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