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Infectious cDNA clones of two strains of Mayaro virus for studies on viral pathogenesis and vaccine development

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ABSTRACT

Mayaro virus (MAYV; family *Togaviridae*, genus *Alphavirus*) is an emerging global threat that can cause severe clinical manifestations similar to Zika, dengue, and chikungunya viruses. Currently, there is a lack of molecular tools to enable a better understanding of the transmission and pathogenesis of MAYV. Here, we detail the development and characterization of infectious clones of two strains of MAYV that produce infectious virus and replicate in mammalian and mosquito cells similarly to wild-type virus. Additionally, clone-derived viruses produced identical infection rates and phenotypes in CD-1 mice compared to the parental strains. This infectious clone system will provide a resource to the research community to analyze MAYV genetic determinants of virulence, determine vector competence, and develop vaccines.

1. Introduction

Arthropod-borne viruses, or arboviruses, produce significant disease burden and economic costs worldwide. Arboviruses such as dengue (DENV), Zika (ZIKV), and chikungunya (CHIKV) are now globally established (Weaver et al., 2018). Since arboviral diseases predominantly affect developing countries, they receive scant attention from the developed world. Without proper monitoring and attention these viruses can cause severe outbreaks, as has recently occurred for CHIKV and ZIKV in the Americas (Weaver et al., 2018). Therefore, it is critical to study emerging pathogens before they produce an outbreak to prevent or control future spread.

One of these newly emerging threats is Mayaro virus (MAYV; family *Togaviridae*, genus *Alphavirus*). MAYV produces a disease that is clinically similar to CHIKV, DENV, and ZIKV, producing fever, arthralgia, and maculopapular rash. Occasionally, MAYV infection leads to chronic polyarthritis, neurological complications, hemorrhage, myocarditis, and even death (Esposito and Fonseca, 2017). First isolated in Trinidad and Tobago in 1954 in the county of Mayaro, the virus has since been reported in many countries across Central and South America, including Brazil, Bolivia, Ecuador, Venezuela, and Haiti, with some imported cases in North America and Europe (Acosta-Ampudia et al., 2018; Levi and Vignuzzi, 2019). In parts of Brazil, MAYV is considered endemic, and outbreaks have occurred periodically since 1955 in many different regions of the country (Esposito and Fonseca, 2017). The increasing incidence of MAYV in areas outside of endemic zones, such as a recent case in Haiti (John Lednicky et al., 2016), has caused concerns that the virus is spreading. Additionally, because of its clinical similarities to other arboviruses, MAYV is often misdiagnosed as CHIKV or DENV, and can occur as a co-infection with other viruses (Acosta-Ampudia et al., 2018; John Lednicky et al., 2016).

Given MAYV's potential to produce an outbreak, it is necessary to devise strategies to prevent further spread. However, the lack of an infectious clone system for MAYV prevents a comprehensive understanding of the transmission, pathogenesis, and immune response to MAYV. Accordingly, we developed and tested infectious clones of two MAYV strains which were readily available through the Center of Disease Control and Prevention (CDC): TRVL 4675 and TRVL 15537, genotype D viruses isolated in the 1950s from a human and a pool of *Mansonia venezuelensis* mosquitos in Trinidad, respectively (Powers et al., 2006). Initially, we produced both strains as infectious clones under an SP6 bacterial promoter. We then transferred the MAYV genome from the SP6-containing clones into a new plasmid backbone under the control of a cytomegalovirus (CMV) promoter and rescued infectious virus to demonstrate their viral growth characteristics in cell culture and a mouse model. The clone-derived strains are similar in both DNA sequence and *in vitro* and *in vivo* replication to the parental strains. Such tools are essential to advancing MAYV research.

2. Materials and methods

Ethics statement. This study was carried out in strict accordance

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with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The research protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech.

Cell Culture. BHK-21, Vero, and LLC-MK2 cell lines were maintained at 37 °C in 5% CO_2. The C6/36 mosquito cell line was maintained at 28 °C in 5% CO_2. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin (herein called DMEM-5). Plaque assays were performed as previously described (Weger-Lucarelli et al., 2017) except that plates were fixed after two days.

Generation of MAYV infectious clones. The MAYV strains TRVL 4675 and TRVL 15537 were a kind gift from Brandy Russell at the Center for Disease Control and Prevention's (CDC) Arbovirus Reference Collection (ARC). The MAYV infectious clones were constructed using a CHIKV backbone containing an SP6 promoter that has been described previously (Coffey and Vignuzzi, 2011). To amplify the MAYV genome, we extracted viral RNA using the Direct-zol RNA Miniprep Plus kit (Zymo Research). We then created cDNA with ProtoScript II Reverse Transcriptase (NEB) using an oligo (dT) primer according to the manufacturer's instructions. We next used the Q5 High-Fidelity 2X Master Mix (NEB) to amplify the genome in three overlapping fragments. The vectors were amplified in the same manner using primers containing 5′ overhangs that overlapped with the 5′ and 3′ end of the viral genome. We then gel purified the PCR products and assembled the DNA using the NEBuilder HiFi DNA Assembly Master Mix (NEB). Next, we transformed the assembly mix into competent NEB Turbo or NEB Stable *E. coli* and screened colonies using restriction digestion following plasmid minipreps with the Zyppy Plasmid Miniprep Kit (Zymo Research). Clone sequences were confirmed by Sanger sequencing. To generate MAYV infectious clones under the control of a cytomegalovirus (CMV) promoter, we used a plasmid backbone that we previously described that is based on pcDNA3.1 (Weger-Lucarelli et al., 2015). To construct these clones, we amplified the entire MAYV genome from the previously described SP6-containing plasmids along with the vector containing overlapping ends using SuperFi 2x master mix (Invitrogen). The sequence of these clones was confirmed using next-generation sequencing (NGS).

Transfection of infectious clone plasmids. We transfected HEK-293T-cells using JetPrime (Polyplus) for recovery of infectious virus from the CMV-based clones. The virus was collected from the transfection supernatant when a 50–75% cytopathic effect (CPE) was observed.

Viral growth kinetics. For BHK-21 and C6/36, cells were seeded at 1×10^6 cells per well, while LLC-MK2 cells were seeded at 2.5×10^5 cells per well, in 12 well plates. Plated cells were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell (back titrations were not performed). Virus stocks were diluted in Roswell Park Memorial Institute medium (RPMI 1640) with 25 mM HEPES and 1% FBS. 100 µl of viral inoculum was added to each well in triplicate and incubated at 37 °C, or 28 °C, in 5% CO_2, for 1 h with rocking every 10 min. After infection, cells were washed 1–2 times with phosphate buffered saline (PBS), and then 500 µl of DMEM-5 was added to each well. We then collected supernatant to determine the baseline amount of infectious virus. Culture supernatants were harvested every 24 h until 50–75% CPE was observed and stored at −80 °C until titration by plaque assay.

***In vivo* growth and pathogenesis in mice**. 3-Week-old female CD-1 mice were obtained from Charles River (Wilmington, MA). Groups of mice (n = 5 for experimental groups, n = 3 for mock treatment group) were infected in the hind-left footpad with 50 µl of 1×10^5 PFU of either parental or clone-derived MAYV. Mice were weighed and monitored daily for signs of disease. Mice were anesthetized using isoflurane and then bled via the maxillary vein each day for three days post infection. We collected blood for only 3 days since in previous studies we did not observe infectious virus at any time point after this (data not shown). Mice were observed for up to two weeks following infection and euthanized when evidence of severe disease was detected. Euthanasia criteria included weight loss greater than 15% of their body weight and evidence of neurological disease such as hind limb paralysis and tremors. Serum was isolated by centrifugation of whole blood samples and titrated using the Vero cell plaque assay.

Real-time RT-PCR. The primer/probe set that was used was obtained from Integrated DNA Technologies (IDT, Coralville, Iowa). The primer sequences used were 100% conserved for both MAYV strains and amplify the nucleotide (nt) region 5028–5127 in nsp3. The primers are as follows: nt5028-Forward - CCTCTGTTAGTCCTGTGCAATAC; nt5127-Reverse - AAGGTGCTTAGGGAGCTACT; nt5060-Probe CACAG TGAAACTACTGTAAGCTTGAGCTCG. The probe has a 5′ FAM reporter and a 3’ ZEN/Iowa Black FQ quencher. A MAYV standard for each strain was prepared by diluting RNA generated with the mMESSAGE mMACHINE kit. Viral RNA in unextracted mice serum samples was quantified using the NEB Luna One-Step RT-qPCR Probe Kit using a CFX96 real-time thermocycler (Bio-Rad). Ct values were fit to the standard curve to generate genome copies/mL of serum.

Statistics. All statistical comparisons were made using GraphPad version 8 (San Diego, CA). Comparisons of viral titers from the growth curve and mouse experiments were performed using a two-way ANOVA with Tukey's correction for multiple corrections and a mixed effects model was performed analyzing weight change. Survival data was compared using the Mantel-Cox test.

3. Results

Construction of MAYV TRVL 4675 and TRVL 15537 infectious clones. A CHIKV infectious clone with a pcDNA3.1 backbone (Invitrogen) was used for construction of the two MAYV clones (Steel et al., 2011; Weger-Lucarelli et al., 2016). The parental viruses were obtained from the CDC and were originally isolated from Trinidad in 1954 (TRVL 4675) and 1957 (TRVL 15537). The sequence of each virus was determined by next-generation sequencing (NGS). Using PCR, we amplified three overlapping viral fragments and the vector was assembled using Gibson Assembly. Each clone possesses a CMV promoter and a 3′ hepatitis delta virus ribozyme to ensure an authentic 3’ untranslated region (UTR) (Fig. 1). One individual plasmid clone was sequenced using NGS for each strain, and one change was identified in each clone compared to the parental virus (Table 1). For the infectious clone for TRVL 4675, we observed a synonymous change at position 2843, and for the infectious clone for TRVL 15537 we observed a single non-synonymous change at genome position 4989, resulting in a lysine to glutamic acid change in the nsp3 gene.

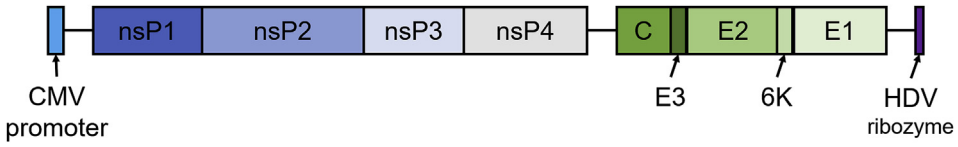

Fig. 1. Genome organization of Mayaro virus (MAYV) infectious clones. The genome of two genotype D MAYV strains, TRVL 4675 and TRVL 15537, were cloned into a pcDNA3.1 plasmid backbone containing a cytomegalovirus (CMV) promoter and a hepatitis delta virus (HDV) ribozyme sequence 5' and 3' to the viral genome, respectively.

Table 1
Sequence differences between clone-derived and parental Mayaro virus (MAYV) strain TRVL 4675 and TRVL 15537.

Virus	Nucleotide position	Nucleic acid in sequence for:		Amino acid change	Gene location
		Parental MAYV	*Clone MAYV*		
TRVL 4675	2843	A	C	None	nsp2
TRVL 15537	4989	A	G	K- > E	nsp3

3.1. In vitro growth properties of parental and clone-derived MAYV strains

To examine the *in vitro* replication kinetics of MAYV strains TRVL 4675 and TRVL 15537, (herein denoted as $4675^{parental}$ and $15537^{parental}$, respectively) and their clone derivatives (herein called 4675^{cloned} and 15537^{cloned}, respectively), we infected several cell lines with each strain at an MOI of 0.1 and measured infectious viral titers by plaque assay (Fig. 2). The replication profile of each parental strain and their respective clones were virtually identical. In C6/36 mosquito cells (Fig. 2A), there were no statistical differences between the parental strain and the infectious clone-derived virus for either MAYV strains at any time point. In BHK-21 mammalian cells (Fig. 2B), a slight replication difference was observed between $4675^{parental}$ and 4675^{cloned} on day 1 post-infection, but this was resolved at later time points ($p = 0.04$ for Day 1 comparison between $4675^{parental}$and 4675^{cloned}). In LLC-MK2 mammalian cells (Fig. 2C), a difference in infectious titers between $4675^{parental}$ and 4675^{cloned} ($p = 0.0053$) and between $15537^{parental}$ and 15537^{cloned} ($p = 0.033$) was seen only on day 3 post-inoculation, past the peak point of replication.

3.2. Pathogenesis of parental and clone-derived MAYV strains in mice

We infected CD-1 mice via the footpad to evaluate the pathogenesis of the $4675^{parental}$ and $15537^{parental}$ strains and their recombinant 4675^{cloned} and 15537^{cloned} derived viruses. Mice infected with the parental strains did not experience statistically different weight change compared to clone-derived virus (Fig. 3A). Additionally, we observed a 100% survival rate for mice infected with $4675^{parental}$ or 4675^{cloned}. An 80% survival rate for mice infected with $15537^{parental}$ and a 40% survival rate for mice infected with 15537^{cloned} (Fig. 3B) all of which were not found to be statistically different. Mice infected with $15537^{parental}$ and 15537^{cloned} experienced symptoms consisting of hunched posture, hind limb paralysis, and significant weight loss starting 4–5 days post infection (dpi).

We collected serum samples on days 1, 2, and 3 post-infection to assess viremia in the mice infected with the two original strains of MAYV and the clone-derived viruses. Infectious viral titers of $4675^{parental}$ and 4675^{cloned} were measurable on dpi 1 and dpi 2, reaching a maximum at 6 $\log_{10}$ PFU/mL but were not detectable at dpi 3 for either viruses except for 1 mouse infected with $4675^{parental}$ which had a viral titer of 2 $\log_{10}$ PFU/mL. Viral titers of $15537^{parental}$ and 15537^{cloned} were measurable at dpi 1, reaching a maximum of 2–3 $\log_{10}$ PFU/mL, and were not detectable at dpi 2 and dpi 3 (Fig. 4A). Viral RNA was measured in serum samples to establish viral infection. Viral RNA was detectable for $4675^{parental}$ and 4675^{cloned} on all days post infection. Viral RNA for $15537^{parental}$ and 15537^{cloned} was only detectable on days 1 and 2 post-infection (Fig. 4B). No statistical differences were observed between the parental and their clone-derived virus at any timepoint for either infectious virus or viral genomes.

4. Discussion

MAYV is an emerging global threat with the potential to cause epidemics similar to what has occurred with CHIKV and ZIKV. Therefore, there is a need for molecular tools to study the replication, transmission, and pathogenesis of the virus to develop strategies to control infection or to design vaccines. While a previous report describes the construction of a MAYV infectious clone used in the construction of a live-attenuated vaccine (Weise et al., 2014), these authors did not present data comparing the clone-derived virus to the parental virus. To this end, we generated infectious clones for two different MAYV strains, TRVL 4675 and TRVL 15537, both isolated in Trinidad in the 1950s (Anderson et al., 1957), and compared their replication in several cell lines and mice. We selected these strains since they were readily available from the CDC and had previously been sequenced. The clone-derived virus for each strain is identical in sequence to the wild type parental virus except for a single nucleotide difference. 4675^{cloned}, the clone-derived virus of strain TRVL 4675, contained a synonymous change at position 2843.15537^{cloned}, the clone-derived virus of strain TRVL 15537, contained a non-synonymous change at position 4989, resulting in a change from lysine to glutamic acid. Neither of the nucleotide changes produced a difference in *in vitro* or *in vivo* virus replication of the clone-derived viruses compared with the parental strains, therefore, the nucleotide changes were not changed to the wild-type sequence. Both clone-derived viruses produced high titers following transfection showing that the plasmids were infectious.

We observed that clone-derived virus was phenotypically similar to their parental strains in growth curve assays in both mammalian and mosquito cell lines. We did not observe statistical differences between clone-derived and parental virus during the exponential phase of growth for C6/36 or LLC-MK2 cells. However, a slight difference ($p = 0.0383$) was observed on day 1 post-infection between clone-derived and parental MAYV TRVL 4675 in BHK-21 cells. Given that no differences were observed in other cell lines or replication in mice, we

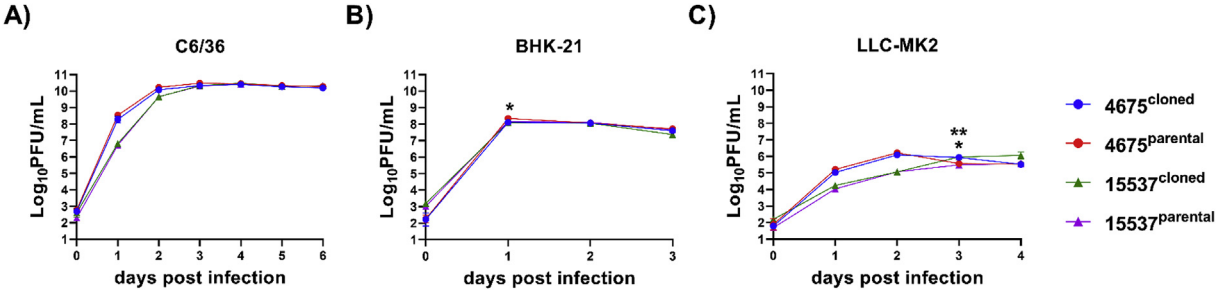

Fig. 2. Growth kinetics of parental and cloned Mayaro virus (MAYV) infectious clones. The growth kinetics of two parental strains of MAYV TRVL 4675 ($4675^{parental}$) and TRVL 15537 ($15537^{parental}$), and their cloned derivatives (4675^{doned} and 15537^{cloned}) was assessed on mosquito (A, C6/36), hamster (B, BHK-21), and nonhuman primate (C, LLC-MK2) cells by infection at an MOI of 0.1 PFU/cell. The cells were infected in triplicate and then supernatant was collected to test by plaque assay daily, including the day of infection. Error bars represent standard deviations from the mean. Statistical analysis was done via two-way ANOVA testing with Tukey's correction for multiple comparisons. On day 1 for BHK-21, there is a statistical difference between $4675^{parental}$ and 4675^{cloned} (*$P < 0.05$). On day 3 for LLC-MK2 there is a statistical difference between $4675^{parental}$ and 4675^{cloned} (**$P < 0.01$) and $15537^{parental}$ and 15537^{cloned} (*$P < 0.05$).

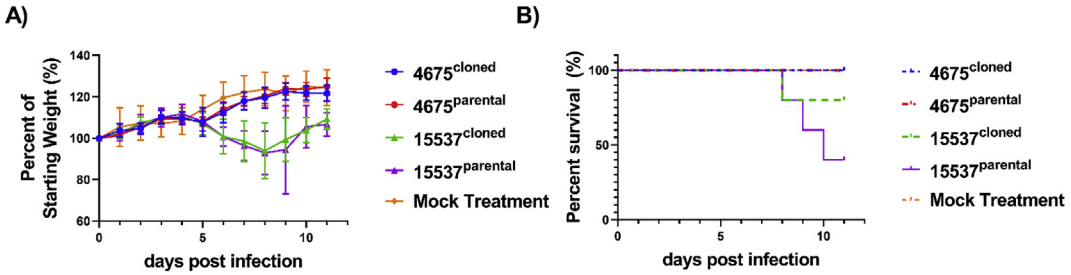

Fig. 3. Pathogenesis of infectious clone-derived MAYV in mice. 3-week-old female CD-1 (n = 5, except for mock which was n = 3) were inoculated with 1×10^5 PFU of either the two parental strains of MAYV, TRVL 4675 ($4675^{parental}$) or TRVL 15537 ($15537^{parental}$), or their cloned derivatives (4675^{cloned} and 15537^{cloned}) in the hind-left footpad and monitored over time. (A) Weight of CD-1 mice post infection, represented as a percentage of starting weight. Statistical analysis was done via a mixed-effects model and there were no statistical differences between weights on any day betwen either parental strains with their corresponding clones. (B) Mortality of CD-1 mice post infection. There was a 100% survival rate for mice infected with $4675^{parental}$ and 4675^{cloned}, an 80% survival rate for mice infected with $15537^{parental}$, and a 40% survival rate for mice infected with 15537^{cloned}. Statistical analysis was done via a Mantel-Cox test and there were no statistical differences between parental and cloned viruses.

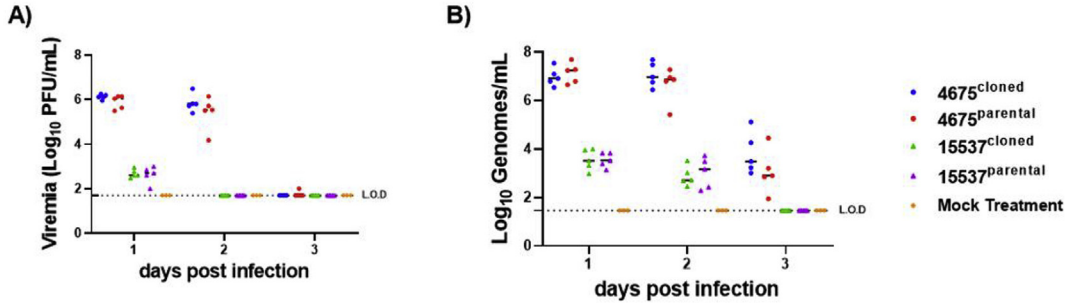

Fig. 4. Replication kinetics of cloned MAYV in CD-1 mice mimics parental MAYV.
3-week-old female CD-1 (n = 5, except for mock which was n = 3) were inoculated with 1×10^5 PFU of either the two parental strains of MAYV, TRVL 4675 ($4675^{parental}$) and TRVL 15537 ($15537^{parental}$), or their cloned derivatives (4675^{cloned} and 15537^{cloned}) in the hind-left footpad. Daily cheek bleeds were performed for 3 days following infection and virus titers in the collected sera were determined by plaque assay in Vero cells (A) and by qRT-PCR (B). Statistical analysis was done via two-way ANOVA testing with Tukey's correction for multiple comparisons and no statistical differences were observed between parental strains and clone viruses. L.O.D indicates the limit of detection for each assay. The L.O.D for panel A is 1.69 $\log_{10}$PFU/mL and for panel B is 1.46 $\log_{10}$Genomes/mL.

can conclude that the replication of the clone-derived and parental strains are sufficiently similar for use in future pathogenesis studies. These data suggest that the clone-derived virus accurately reflects the replication of the parental virus in both vertebrate and invertebrate cell lines.

To test pathogenesis and assess vaccine efficacy, productive infection of mice is necessary. We, therefore, infected immunocompetent CD-1 mice and observed statistically similar levels of viremia, weight loss, and mortality between parental and clone-derived MAYV strains, demonstrating that the clone-derived viruses have similar characteristics in mice. Mice infected with either $15537^{parental}$ or 15537^{cloned} exhibited greater mortality rates due to perceived neurovirulence, which resulted in hind limb paralysis, weakness, and incoordination. The neurologic symptoms of TRVL 15537 have been previously documented as a difference compared to TRVL 4675, however, the mechanism of this occurrence is unclear (Aitken et al., 1960). Additionally, neurovirulence has been shown to occur in alphaviruses, specifically in cell culture-adapted laboratory strains, due to heparan sulfate binding can increase neuroinvasiveness (Ryman et al., 2007). TRVL 4675 has been passaged seven times in suckling mice and once in BHK-21 cells. The passage history of TRVL 15537 is not entirely clear, as the CDC lists 4 passages in an unknown host, one in suckling mice, and one in Vero cells. How Aitken et al. report that the virus was isolated in suckling mice and that additional passages occurred in either suckling mice or hamster kidney cells (Aitken et al., 1960). It is unclear what role the passage history of each virus has on the pathogenesis observed in CD-1 mice.

While we observed infectious virus in all the MAYV-infected mice on day 1 post-infection, both clone-derived and parental TRVL 15537 titers were very low at this time point and were not detectable thereafter. To ensure that the mice were infected, we confirmed the levels of MAYV RNA in the blood and found detectable levels for all MAYV-infected groups on days 1–2 post-infection. These data suggested that all the mice were productively infected albeit the level of replication was strain specific. Given that no differences were observed between the parental and clone-derived viruses in the level of infectious virus or RNA in the serum at any day post-infection, the infectious clones described here represent valuable tools for studying MAYV pathogenesis in future studies.

In summary, our results show that the strains of MAYV tested here efficiently replicate in cell culture and productively infect immunocompetent mice. We found that the virus derived from the infectious-clone plasmids replicated simielar to the parental virus in a variety of cell lines. We also show that each clone-derived virus behaves similar to its parental counterpart following infection of outbred CD-1 mice in terms of viremia, serum RNA levels, weight-loss, and mortality. These data suggest that clone derived MAYV will be useful for mapping genetic determinants of pathogenesis and can provide a starting point for the generation of a recombinant vaccine.

Acknowledgments

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