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Studies on Isolation and Characterization of Alphaviruses

Sindbis Virus and Eilat Virus

identified from mosquitoes

in Zambia

(ザンビアの蚊から単離されたアルファウイルス属
シンドビスウイルスおよびエイラットウイルスの
性状解析)

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Abbreviations

aa	Amino acids
BLAST	Basic Local Alignment Search Tool
CNS	Central nervous system
CI	Confidence interval
CPE	Cytopathic effect
DDBJ	DNA Data Bank of Japan
DMEM	Dulbecco's modified Eagle medium
dpi	Days post infection
EILV	Eilat virus
EMBL	European Molecular Biology Laboratory
EMEM	Eagles minimum essential medium
ERES	Excellence in Research Ethics and Science
FBS	Fetal bovine serum
HAI	Haemagglutination inhibition
hpi	Hours post infection
IFA	Immunofluorescent assays
IFN	Interferon
MIR	Minimum infection rate
MOI	Multiplicity of infection
MWAV	Mwinilunga alphavirus
NGS	Next generation sequencing
nt	Nucleotides
PFU	Plaque forming unit
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
RACE	Rapid Amplification of complementary DNA Ends
RT-PCR	Reverse-transcriptase polymerase chain reaction
SINV	Sindbis virus
SC	Subcutaneous
SD	Standard deviation
sp.	Species
TCID ₅₀	50% tissue culture infectious dose
WEE	Western equine encephalitis

Notes

Chapter I and II

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General Introduction

Alphaviruses are positive sense single-stranded RNA viruses transmitted by mosquito vectors which infect a variety of hosts including humans, and cause a wide variety of clinical symptoms in patients. They are members of the family *Togaviridae* and genetically encode five structural proteins (Capsid, E1, E2, E3 and 6K) and four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4). While several alphaviruses are associated with human disease, the viruses remain poorly understood in terms of their host specificity mechanisms, evolution and pathogenesis (1). Sindbis virus (SINV) belongs to an alphavirus species within the Western Equine Encephalitis (WEE) complex and are found in the Old World. Eilat virus (EILV) is an alphavirus recently identified in mosquitoes and forms a distinct group from other known alphavirus complexes (2). SINV travels between continents by migratory birds and infects humans and other mammals via ornithophilic mosquitoes, whereas EILV has a very narrow host range and restricted mosquito vectors. In this study, SINV and EILV identified during alphavirus screening of Zambian mosquitoes were isolated and characterized. A fundamental understanding of the biological properties of these two alphaviruses is crucial for elucidating unknown aspects of the genus *Alphavirus*. It also plays an important role in uncovering the mechanism driving their global spread and emergence in new regions as well as the factors determining host preference. Such findings are useful for developing effective control strategies for alphaviruses from a global perspective.

SINV causes acute human infections characterized by rash, fever, headache and fatigue; whereas persistent infection by SINV often leads to long-term musculoskeletal disorders accompanied by widespread joint pain (3). Although migratory birds serve as the main amplifying hosts of SINV, they do not produce any signs of illness despite carrying significant amounts of the virus (4). After being first isolated from *Culex* species (sp). in Egypt in 1952, SINV was subsequently identified from *Culex pipiens*, *Culex univittatus* (5), *Culex torrentium* (6), *Culex morsitans* (7), *Aedes* sp. (8) and *Anopheles* sp. and others (9). SINV has been confirmed across the African continent and other areas in the Eastern Hemisphere (10-12). There are six recognized genotypes of SINV (Genotype 1-6). Genotypes 2, 3, 5, and 6 are found in Australia, India, New Zealand, and Australia, respectively (13, 14). Meanwhile, Genotype 1 has been detected in Central and South Africa, Northern Europe and the Middle East, and Genotype 4 has been reported in Azerbaijan and Western China. Of the 6 genotypes, only Genotype 1 causes human disease (3, 13). While a reintroduction of SINV has been confirmed into Central Africa, not enough research has been conducted in Zambia

despite the presence of relevant mosquito vectors and intermediate hosts (15-17). To date, it has been reported that one case among 40 human sera from German aid workers in Zambia was positive for anti-SINV IgG by using in-house indirect immunofluorescence assays (IFA) (18).

Analysis of the pathogenicity of SINV has been carried out using mouse models, such as suckling mice and immunodeficient mice. Previous studies have revealed that interferon (IFN)- α , - β , and - γ play important roles in protecting mice from fatal SINV infection (19-22). In two of these studies, mice lacking type I IFN- α/β receptor (A129 mice) or type I and II IFN- $\alpha/\beta/\gamma$ receptor (AG129 mice) died within 3 days post infection (dpi) when infected with 100 plaque forming units (PFU) of SINV, whereas immunocompetent mice (129 SvEv) infected under the same parameters showed no morbidity or significant weight loss (19, 20).

EILV is an insect-specific alphavirus first isolated from *Anopheles coustani* mosquitoes in Israel (2, 23). It was subsequently detected in *Culex pipiens* in Morocco and isolated from *Culex univittatus* mosquitoes in Namibia (24, 25). EILV exhibits host specificity and has been confirmed to replicate only in mosquito cells, but not in mammalian or avian cells (2, 26). Phylogenetically, it is closely related to the viruses belonging to the Western equine encephalomyelitis (WEE) virus complex. EILV forms a group with Mwinilunga alphavirus (MWAV) identified in Zambian mosquitoes in 2016 (16). A linear discriminant analysis based on dinucleotide ratios suggest that MWAV is an insect-specific alphavirus.

In Chapter I, a new strain of SINV was isolated during alphavirus screening in Zambia and characterized. The pathogenicity of the Zambian SINV was assessed in immunodeficient as well as immunocompetent mouse models in comparison with reference SINV strain AR339.

In Chapter II, a newly isolated EILV strain from *Culex* mosquitoes in Zambia was characterized in terms of its genetic relation in alphaviruses and replication in mosquito and mammalian cells, thus it is an important work for understanding the dissemination of alphaviruses across major geographic regions.

Chapter I: Isolation and characterization of Sindbis virus from mosquitoes captured in Zambia

Summary

As a result of alphavirus screening in mosquitoes captured in Zambia, SINV was isolated and designated as strain zmq_17M115 (SINV M115). This SINV belongs to Clade D of SINV Genotype 1, a type that is prevalent in Europe, the Middle East, and Africa (12) . This finding is important from a global epidemiological standpoint as it suggests that SINV may have been introduced to Zambia from neighboring regions via birds serving as vectors or amplifying hosts. The growth efficiency of SINV M115 in mammalian cells, such as BHK-21 cells was similar as that of the prototype SINV AR339; however SINV M115 proliferated more rapidly than SINV AR339 in mosquito-derived C6/36 cells. Pathogenicity of SINV M115 was compared with that of SINV AR339 using type I/II IFN receptor deficient AG129 mice. *In vivo* experiments revealed that both SINV strains caused lethal infection of AG129 mice within 3 days post infection (dpi) inoculated by subcutaneous (SC) route using 10-1,000 plaque forming units (PFU) of the viruses. However, there was no change of body weight and survival rate in C57BL/6 mice inoculated with 1,000 PFU of both SINVs compared to mock infected group. Thus, the SINV M115 was also found to be highly pathogenic in AG129 mice. This study indicates the possible circulation of SINV in Zambia and highlights the need for surveillance of potential epidemics in humans.

Introduction

SINV shows a high seroprevalence approaching 40% in humans in endemic areas of some Northern European countries (27, 28). On the African continent, SINV has been isolated from specimens of febrile patients in Uganda and South Africa. Outbreaks have been reported in Egypt (29) and South Africa (30), with serological tests. A serosurvey conducted in Uganda reported a SINV antibody prevalence of 45.1% among children under 15 years age and 77.3% among adults in the north-western region of the country (31). In South Africa, seroepidemiological data on SINV infection was reported from 2006 to 2010, and 3,631 specimens from human cases with suspected arboviral infections were tested. Anti-SINV IgM antibodies were detected in 1.3% (21/1,606) of serum specimens from 2006 to 2009 and in 10% (208/2,025) of serum specimens in 2010 (30).

In Zambia, the mosquito-borne viruses reported to date include members of the genera *Orthoflavivirus*, *Alphavirus*, and *Phlebovirus* (32). In 2020, seroprevalence of alphaviruses in Zambia was assessed using IgG Enzyme-Linked Immunosorbent Assay in 214 participants from the Lukanga swamp area, revealing seropositive rate of 36.9% for Chikungunya virus, and 19.6% for Mayaravirus (33). In contrast, another medically important alphavirus O'nyong-nyong virus, which is endemic in several Sub-Saharan African nations has not been reported in Zambia. However, mosquitoes of the genus *Anopheles*, which are primary vectors for O'nyong-nyong virus, are present in Zambia (34). Seroprevalence data on SINV infection in Zambia has been reported in one case of Germans working in Zambia from 1987 to 1993 (18). Orthoflavivirus surveys in Zambia have identified West Nile virus from *Culex* mosquitoes (15) and farmed crocodiles (35). Such cumulative findings thus signify that ongoing surveillance is important for estimating risk of arbovirus exposure in Zambia and neighboring countries.

Materials and Methods

Ethics statement

All animal experiments were approved by the Animal Care and Use Committee of Hokkaido University (Approval numbers: 23-0063 and 23-0178) and were performed in accordance with the committee's guidelines. The handling of pathogens was carried out in accordance with the provisions of Hokkaido University Biosafety Management Regulations on Pathogens and Other Hazardous Agents under the experimental plan approved by a committee [Approval number 14(17)].

Cells and viruses

C6/36 (CRL-1660, American Type Culture Collection: ATCC, Manassas, VA) and BHK-21 (RCB1423, RIKEN Bioresource Research Center: BRC, Tsukuba, Japan) cells were maintained in Eagle's Minimum Essential Medium (EMEM) containing 10% Fetal Bovine Serum (FBS) under 5% CO₂ at 28 °C. Vero cells (VERO C1008, CLR-1586) were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS under 5% CO₂ at 37 °C. The SINV AR339 strain (VR-1248) was obtained from the ATCC.

Mosquito collection and alphavirus screening

Mosquito surveillance was conducted in the Western part of Zambia (Mongu district) between 2017 and 2019 with permission from the Excellence in Research Ethics and Science (ERES) converge ethics committee (Ref. No. 1382-2020) (15).

In total, 5,843 female mosquitoes were captured and divided into 329 pools by species (maximum 40 mosquitoes per pool). The pooled mosquitoes were homogenized in EMEM with 2% FBS. A part of the homogenate was subjected to RNA extraction with Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA). Pan-alphavirus Reverse transcriptase polymerase chain reaction (RT-PCR) screenings were conducted as described previously (16). In brief, RT-PCR was performed with PrimeScript One Step RT-PCR Kit VER.2 (Takara Bio, Shiga, Japan) using pan-alpha primer sets, such as, nsP4-6692 F (5'-CAYACRYTRTTYGAYATGTCDGC-3') and pan-alpha nsP4-7152 R (5'-GCRTCDATKATYTTBACYTCCAT-3') with the following conditions: 50 °C for 30 min, 94 °C for 2 min, 43 cycles of 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 5 min 60 °C. The PCR products (approximately 460 bp) were visualized on agarose gels stained with

ethidium bromide and purified on 2% (w/v) low melting SeaPlaque agarose (Lonza, Sydney, Australia) and the MonoFas DNA Purification Kit I (GL Sciences, Tokyo, Japan). The sequence of the purified PCR products were analyzed by Sanger sequencing using Big Dye Terminator v3.0 Cycle Sequencing kit (ThermoFisher Scientific, Waltham, MA, USA) using the ABI Genetic Analyzer 3500 XL (ThermoFisher Scientific) and analyzed by Basic Local Alignment Search Tool (BLAST: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Virus isolation and whole genome sequencing

Either C6/36 or BHK-21 cells were inoculated with the homogenates positive for pan-alphavirus RT-PCR and cultured for one week. After the second passage of the homogenate-inoculated cells (C6/36 or BHK-21), the supernatants from the cells showing a cytopathic effect (CPE) were subjected to cloning by limiting dilution methods. After 3-6 passages of the cells, the supernatants were subjected to RNA extraction with Direct-zol RNA Miniprep kit. Whole genome sequencing of the purified RNA samples was conducted through the Illumina Miseq platform (Illumina, San Diego, CA, USA) using Nextera XT DNA Library MiSeq Reagent Kit v3 (CLC bio, Quiagen, Hilden, Germany). The terminal sequence of the viral genome was determined by the 5'- and 3'- rapid amplification of cDNA ends (RACE using SMARTer RACE 5'/3' kit (Takara Bio) to obtain the terminal sequences as described previously (16). SINV isolation was performed by inoculating the lowest dilution of cell culture supernatant that caused CPE in the limiting dilution assay into BHK cells. The supernatant was carefully removed and stored in 300 µL aliquots at -80 °C.

qRT-PCR screening for SINV

Quantitative RT-PCR (qRT-PCR) screening was performed on all the mosquito pools using the One Step TB Green PrimeScript PCR Kit II (Takara Bio) on a QuantStudio 3 real-time PCR system (ThermoFisher Scientific) with the following protocol (42 °C for 5 min, 95 °C for 10 sec, 40 cycles of 95 °C for 3 sec 60 °C for 30 sec) using the primer sets as follows; zSINV q466F (5'-GCTTCCACAACGATGTTACCT-3'), SINV 603R (5'-GTCGAAGCCAATCCAGTACA-3'). The minimum infection rate (MIR) was calculated by maximum likelihood estimation in the Pooled Infection Rate version 4.0. (Centers for

Disease Control and Prevention, Fort Collins, Colorado, USA) (36). The confidence interval (CI, 95%) was indicated in the range between the upper and lower bounds.

Phylogenetic analysis

Genomic sequences of 54 reference strains of SINV were obtained from GenBank. Multiple nucleotide sequence alignment of coding regions was constructed with SINV_zmq17_M115 (LC869668) and 54 reference strains of SINV by ClustalW in MEGA 11 software (Pennsylvania State University, Penn State, USA) (37). Phylogenetic trees were constructed by the Maximum likelihood method using GTR+G+1 model with 1,000 bootstrap replicates.

***In vitro* growth kinetics assay**

The PFU of SINV zmq_17M115 initial stock was determined by the plaque assay using Vero cells. Vero cells seeded in 12-well plates were inoculated with serially diluted culture supernatants for 1 h with intermittent rocking at 37 °C and then overlaid with DMEM containing 2% FBS and 1% methyl cellulose in each well. After 48 hrs, the cells were fixed with 10% formalin and stained with 1% crystal violet. At the time of infection, the number of cells in each well to be infected was calculated, and the multiplicity of infection (MOI) was determined using the formula $MOI = PFU/cell\ number$.

For the growth kinetics assay, Vero or BHK-21 cells in 6-well plates were infected with SINV at an MOI of 0.0001, and similarly C6/36 cells in 6-well plates were infected with SINV at an MOI of 0.01. Culture supernatants were harvested at different time points and subjected to titration by plaque assay.

The statistical analysis of growth curve difference between two strains were performed by two-way ANOVA with Bonferroni post-hoc test using Prism 9 (GraphPad software, Boston, MA).

***In vivo* analysis of SINV pathogenicity**

All animal experiments were conducted in a BSL-2 laboratory at the International Institute for Zoonosis Control, Hokkaido University under the above-described ethical approval. AG129 mice (4-weeks-old, n=3/group) were subcutaneously inoculated with 50 µL

of 1 or 10, PFU of SINV zmq_17M115 or AR339. Similarly AG129 mice (8-weeks-old, n=3/group) were inoculated subcutaneously with 50 μ L of 1,000 PFU of SINV zmq_17M115 or AR339 and a control group was inoculated with MEM 2% FBS. Male C57BL/6 mice (3-weeks-old, NCrSlc, Japan SLC, n=4/ group) were similarly inoculated with 1,000 PFU of SINV zmq_17M115 or AR339 by subcutaneous method. Mice were monitored daily for body weight changes and survival for 7 or 14 dpi. Percentage body weight change over time and Kaplan-Meier survival curves were analyzed by Prism 9 (GraphPad software).

Results

Isolation of alphaviruses from *Zambian mosquitoes*

For surveillance of alphaviruses in mosquitoes captured in Zambia, viral RNA screening and virus isolation were conducted. Initial screening using pan-alphavirus RT-PCR detected positive signals in a pool of *Culex univittatus* (zmq17_M115, 31 mosquitoes/pool) captured in the Mongu district in 2017 (Table 1), and the sequence of the PCR product was similar to the genome of SINV (Table 1). SINV-specific qRT-PCR was subsequently employed on all the mosquito pools collected in Mongu area to check the prevalence of SINV. The zmq17_M115 pool was positive for SINV with an MIR of 0.42 (95% CI: 0.02-2.02) in 746 *Culex* mosquitoes (Table 1). Based on the results of the RT-PCR, the zmq17_M115 pool was used for virus isolation. Lysates of SINV-qRT-PCR positive mosquito pools were inoculated to mosquito-derived BHK-21 cells. The cytopathic effect (CPE) was observed from the second passage in both cell lines. SINV in the supernatants were passaged in BHK-21 cells. Supernatants containing viruses were used for subsequent analysis.

Whole genome sequencing of isolated *Zambian SINV*

The whole genome sequences of the *Zambian SINV* (strain zmq17_M115, SINV M115) was determined using next generation sequencing (NGS) and RACE analyses. The genome sequences (11,709 nt) of SINV M115 has been submitted to the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank databases under the accession numbers LC869669. SINV M115 shares 98% nucleotide sequence identity to SINV BONI_566_KENYA_2013 strain (accession no. KY616987) identified from *Aedes ochraceus* mosquito in Kenya (Table 2). The *Zambian M115* had >98% amino acid sequence similarities to the reference sequence of SINV (accession no. NC_001547) and Kenyan SINV strains in all viral proteins with exception of nsP3. This reference SINV strain (NC_001547) has 99.79% nucleotide coding sequence homology to the prototypic SINV AR339 used in this study. M115 nsP3 exhibited relatively low sequence identities (<96.4%) with known SINV strains (Table 2).

Phylogenetic analysis and genotype assignment

SINV strains consists of 6 genotypes and have an area-specific evolutionary history (13). The phylogenetic analysis of SINV revealed isolated Zambian SINV M115 belongs to the Genotype 1 clade D forming a cluster with SINV isolates from Kenya, South Africa and Algeria collected from 2013 to 2017 (Fig. 1). Other clusters in the clade D also included the prototype AR339 strain from Egypt and SINV isolates from Middle Eastern countries (Israel and Saudi Arabia), as well as Southern and Central Europe (Slovak Republic, Italy), and most of them were found before 1990. The discovery of SINV in Zambia indicated that the distribution of clade D SINVs were expanding widely across Africa in recent years.

Growth kinetics of the isolated Zambian SINV

The growth properties of Zambian SINV M115 were compared with reference SINV strain AR339. Both SINV M115 and AR339 attained a saturating viral titer of 10^8 PFU/mL at 36 hpi in Vero and BHK-21 cells (Figs. 2A and B). AR339 reference strain had relatively high growth property in these mammalian cell lines compared to Zambian M115. In this experiment, the amounts of SINVs for inoculation are different in mammalian and mosquito cell lines; 0.0001 MOI for Vero and BHK-21 cells, and 0.01 MOI for C6/36 cells. In C6/36 cells, the growth of SINV M115 reached 10^8 PFU/mL at 72 hpi and was statistically higher than that of AR339. The growth rate of both SINV strains in C6/36 cells was slower than that of mammalian cells, consistent with a previous report (38, 39) (Fig. 2C). These results suggest that the newly isolated SINV M115 strain, obtained from mosquitoes, exhibits greater proliferative properties in mosquito cells than the laboratory-adopted SINV AR339.

***In vivo* analysis of SINV pathogenicity**

The pathogenicity of isolated SINV was analyzed by using AG129 mice without type I/II IFN receptors and immunocompetent C57BL/6 mice. In AG129 mice, all mice infected with 1,000 and 10 PFU of either SINV M115 or AR339 via subcutaneous (SC) route died by 3 dpi. (Figs. 3 and 4). In mice infected with 1 PFU of either SINV M115 or AR339, all mice (n=5) except one inoculated with SINV M115 survived after infection without significant body weight loss (Fig. 5). In contrast, there was no pathogenicity in C57BL/6 mice inoculated with 1,000 PFU of SINV M115 or AR339 via SC route as they showed no weight

loss and had a survival rate of 100% (Fig. 6). These results suggest that Zambian SINV M115 exhibits high pathogenicity in immunodeficient AG129 mice, similar to the reference strain SINV AR339. It is known that interferon production is one of the main immune responses to arboviral infection so the pathogenicity of these viruses in AG129 mice is consistent with the expected outcome (19).

Table 1: Screening results for alphaviruses in mosquitoes captured in Mongu, Zambia.

Year Month	Pool no.	Mosquito no.	Mosquito species	Pan-alpha RT-PCR Positive number	SINV qRT-PCR positive number (Ct)	EILV qRT-PCR positive number (Ct)	MIR (95% CI)*	Virus isolate (Accession no.)
2017 May	49	746	<i>Culex spp.</i>	1	1 (22.3)	0	0.42 (0.02-2.02)	SINV zmq17_M115 (LC869668)
	4	11	<i>Aedes spp.</i>	0	0	0		
	9	62	<i>Coquillettidia spp.</i>	0	0	0		
	8	132	<i>Mansonia spp.</i>	0	0	0		
	1	1	<i>Uranotaenia spp.</i>	0	0	0		
	2	10	<i>Aedeomya spp.</i>	0	0	0		
	65	1,444	<i>Anopheles spp.</i>	0	0	0		
2018 Aug	19	314	<i>Culex spp.</i>	0	0	0		
	1	2	<i>Anopheles spp.</i>	0	0	0		
	1	1	<i>Coquillettidia spp.</i>	0	0	0		
2018 Dec	39	874	<i>Culex spp.</i>	0	0	0		
	3	6	<i>Aedes spp.</i>	0	0	0		
	11	102	<i>Coquillettidia spp.</i>	0	0	0		
	16	392	<i>Mansonia spp.</i>	0	0	0		
	1	3	<i>Uranotaenia spp.</i>	0	0	0		
	1	1	<i>Aedeomya spp.</i>	0	0	0		
	7	56	<i>Anopheles spp.</i>	0	0	0		
2019	37	871	<i>Culex spp.</i>	1	0	1 (13.0)	0.74 (0.04-3.55)	EILV zmq19_M44 (LC869669)
	7	21	<i>Aedes spp.</i>	0	0	0		
	11	80	<i>Coquillettidia spp.</i>	0	0	0		
	9	174	<i>Mansonia spp.</i>	0	0	0		
	1	1	<i>Uranotaenia spp.</i>	0	0	0		
	1	1	<i>Aedeomya spp.</i>	0	0	0		
	26	538	<i>Anopheles spp.</i>	1	0	1 (19.5)		
							1.51 (0.09-7.02)	
Total	329	5,843		3	1	2		

*Minimum infection rates (with 95% Confidence interval) of EILV or SINV

Table 2

Nucleotide and Amino acid homology of SINV isolate zmq17 M115 to closely related SINV isolates

Sequence identity to Zambian Sindbis virus isolate zmq17 M115 (%)										
Strain	Nucleotide	Protein								
	Whole genome	nsP1	nsP2	nsP3	nsP4	Capsid	E3	E2	6k	E1
	11,709 nt	540 aa	807 aa	549 aa	616 aa	264 aa	63 aa	421 aa	55 aa	438 aa
Sindbis virus isolate										
BONI_584_KENYA_2013	96.49	99.63	98.88	96.37	99.35	98.86	98.44	98.82	100	99.32
KY616985.1										
Sindbis virus isolate										
BONI_566_KENYA_2013	97.96	99.63	98.88	96.37	99.03	98.86	98.84	98.82	100	99.32
KY616987.1										
Sindbis virus reference isolate										
NC_001547.1	96.46	98.7	98.2	95.46	99.03	99.62	99.44	98.11	100	99.34
nt: nucleotides, aa: amino acids										

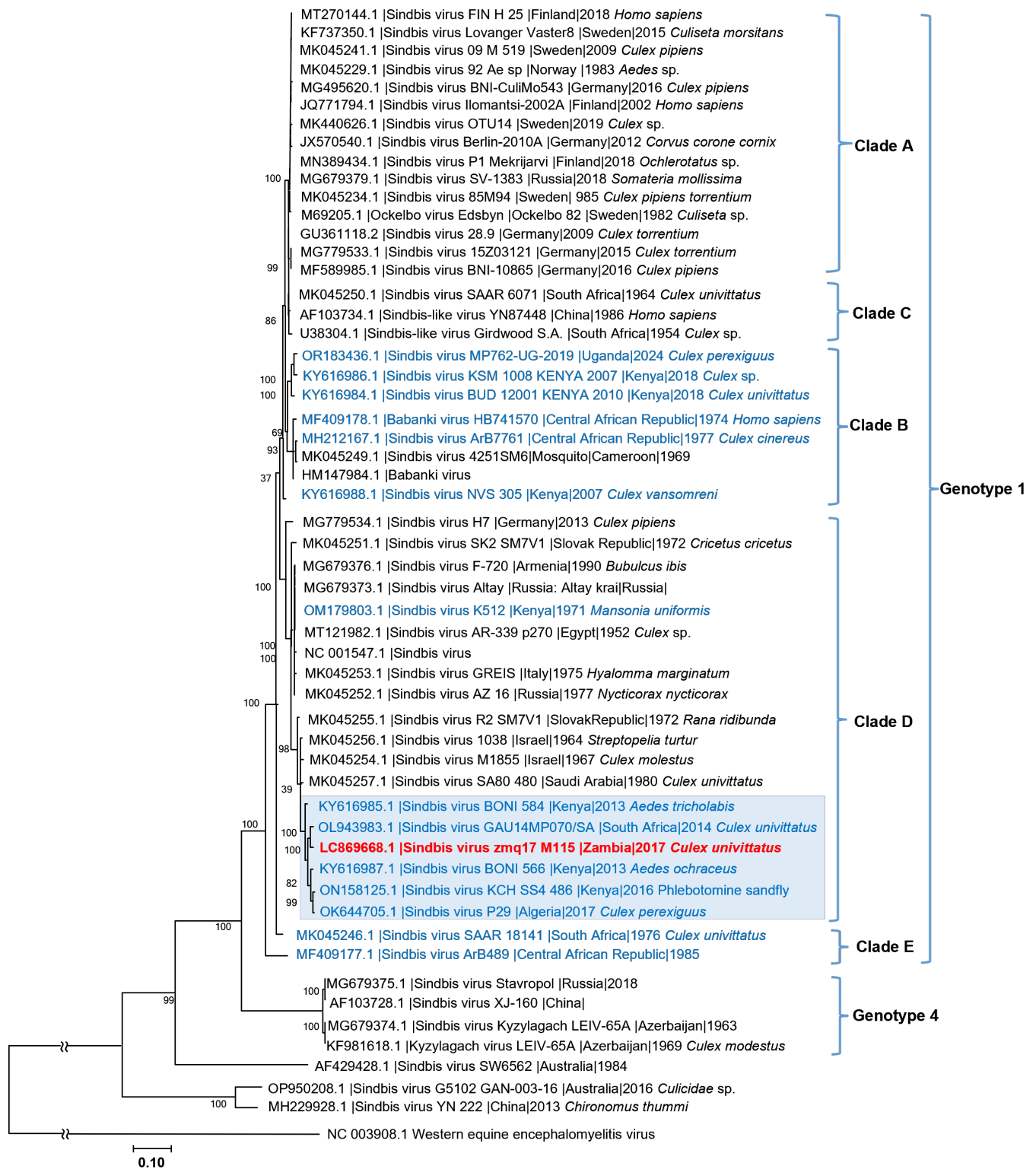


Fig. 1 Phylogenetic analysis based on coding sequences of SINV.

The isolated Zambian SINV M115 in this study is highlighted in red. SINV strains (n=54) were obtained from GenBank and included in the analysis. The phylogenetic tree was constructed by the Maximum Likelihood method using a GTR+G+1 model with 1,000 bootstrap replicates. Genotypes are shown to the right side of the tree. The bootstrap values are shown at the branch node of each clade. The scale bars represent the number of substitutions per site. Red text indicates Zambian SINV M115 and isolated in this study, and blue text indicates African SINV isolates. The cluster including SINV M115 in clade D is highlighted in light blue.

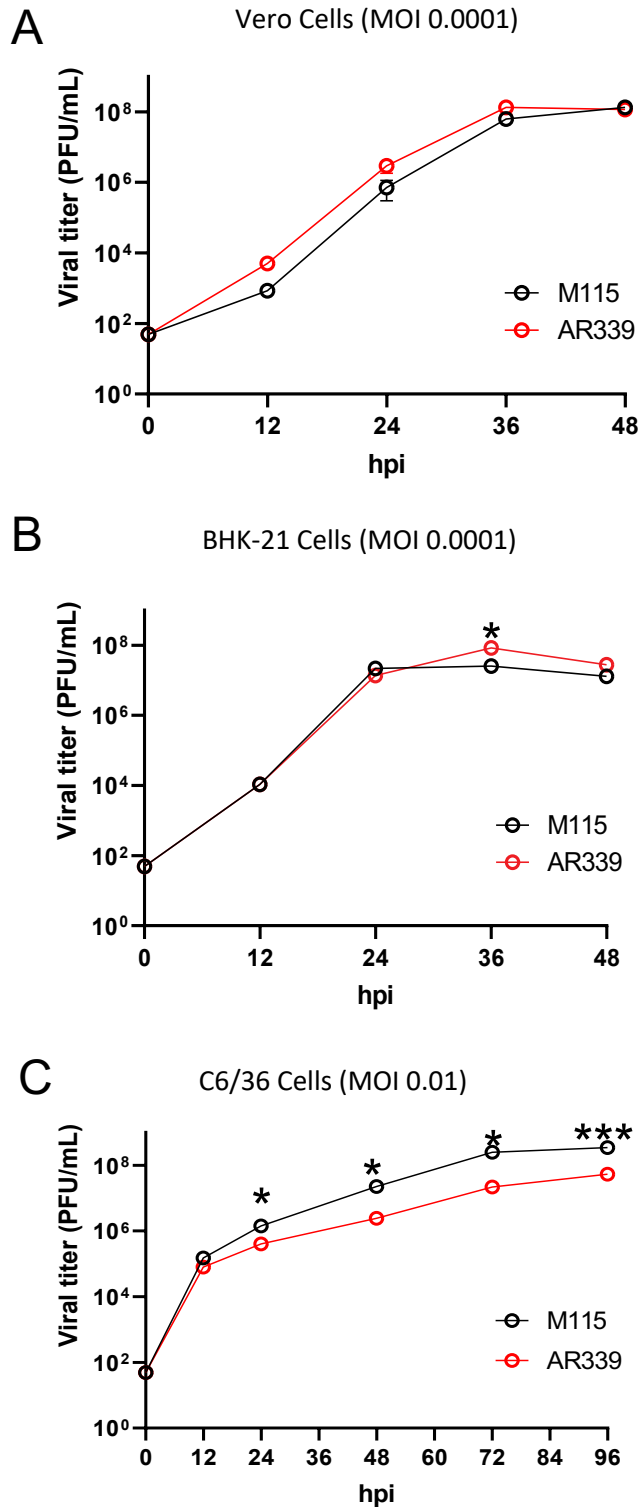
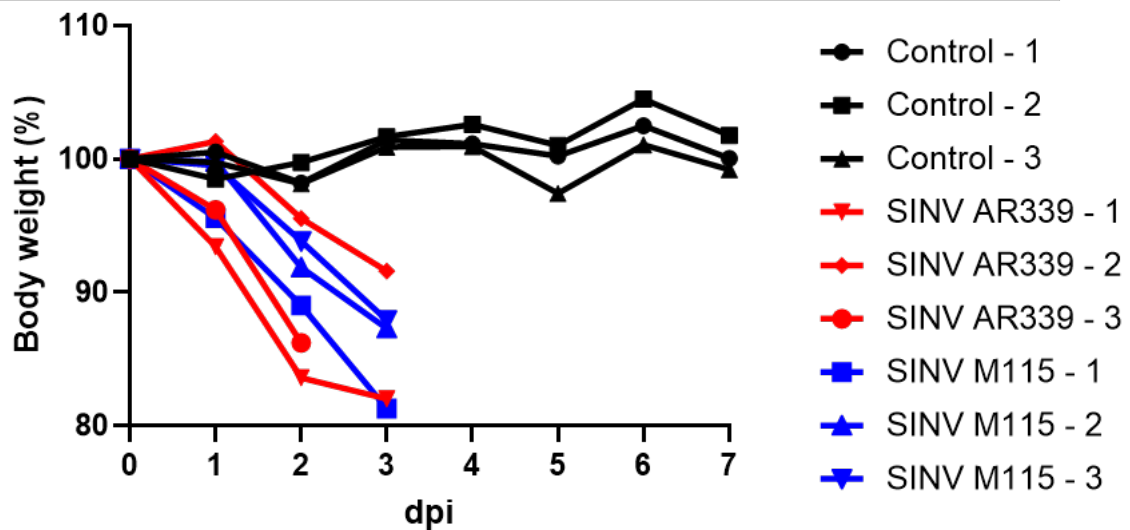


Fig 2: Growth kinetics of Zambian SINV isolate M115.

Vero (A) and BHK-21 (B) cells were infected with SINV isolate M115 and AR339 at an MOI of 0.0001. C6/36 cells (C) were infected with SINV at an MOI of 0.01. Titters at the indicated each time point were determined by plaque assay. The values shown are mean $\pm$ standard deviation (SD) of triplicate. * $p < 0.05$, *** $p < 0.001$ by two-way ANOVA test.

A

AG 129 mice (1,000 PFU)



B

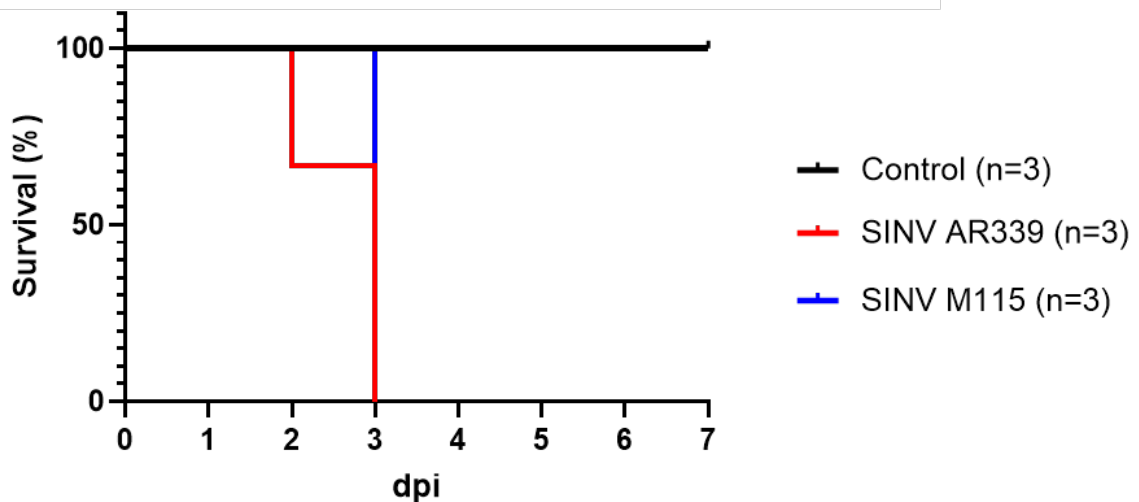
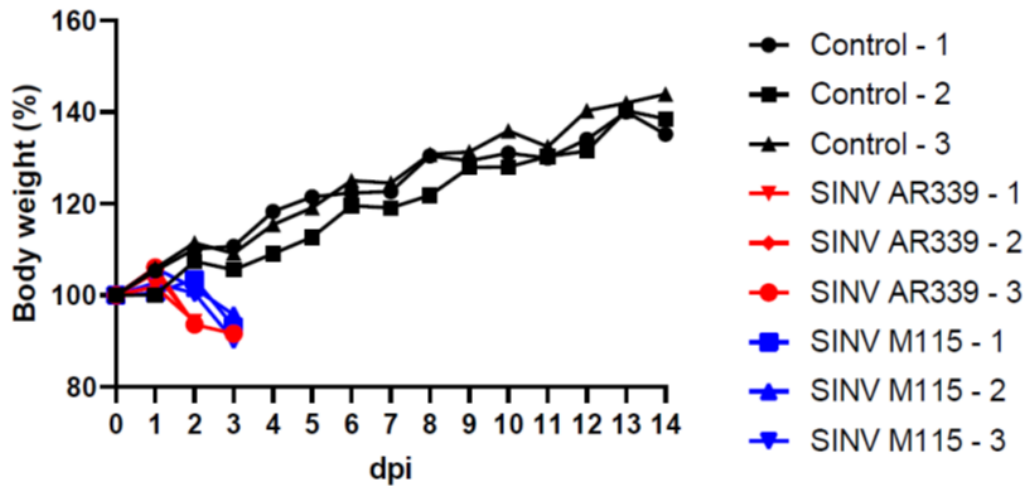


Fig. 3: Body weight change graph (A) and survival curve (B) of AG129 mice infected with 1,000 PFU of SINV

AG129 interferon knockout mice (n=3 per group) were inoculated SC into the nape of the neck with 50 μ L of 1,000 PFU of SINV M115 or AR339 and a control group was inoculated with MEM 2% FBS. Healthy immunocompetent B6 mice (n=4 per group) were similarly inoculated SC with 1000 PFU of SINV M115 or AR339. All mice were monitored daily for body weight changes and survival up to 7 dpi. Percentage body weight change vs. time and Kaplan-Meier survival curves were plotted in Prism 9 (GraphPad software).

A

AG129 mice (10 PFU)



B

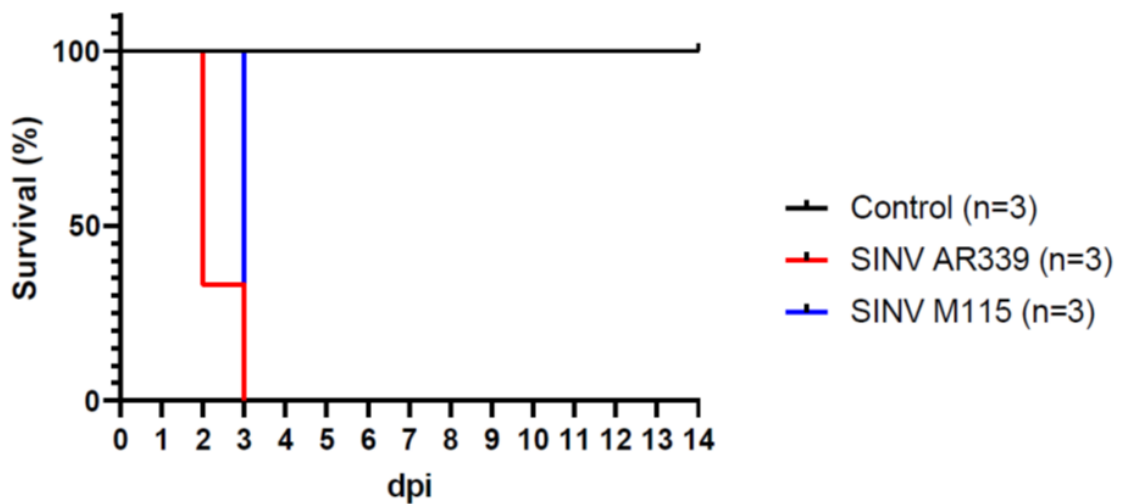
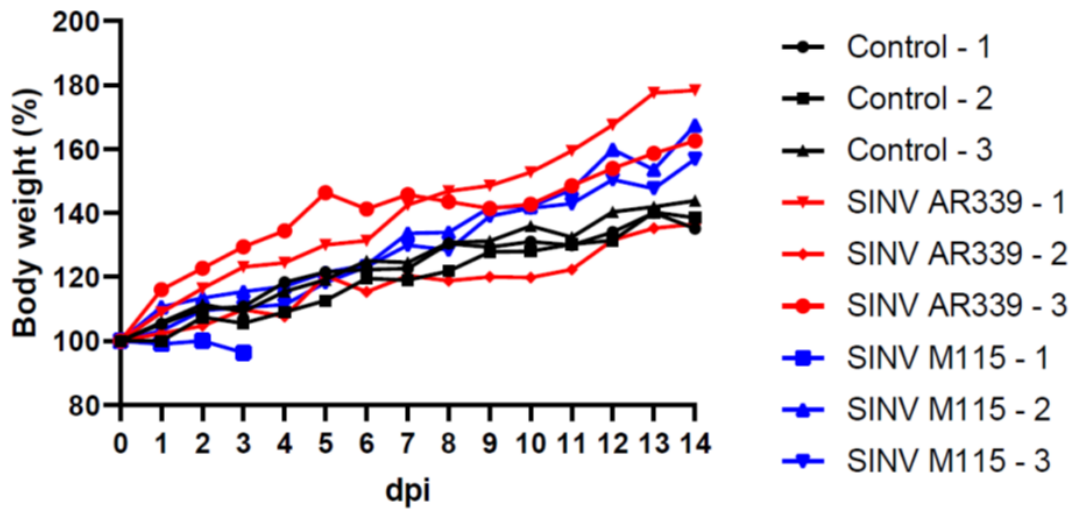


Fig. 4: Body weight change graph (A) and survival curve (B) of AG129 mice infected with 10 PFU of SINV

AG129 interferon knockout mice (n=3 per group) were inoculated SC into the nape of the neck with 10 PFU of SINV M115 or AR339 and a control group was inoculated with MEM 2% FBS. Mice were monitored daily for body weight changes and survival up to 14 dpi. Percentage body weight change vs. time and Kaplan-Meier survival curves were plotted in Prism 9 (GraphPad software).

A

AG129 mice (1 PFU)



B

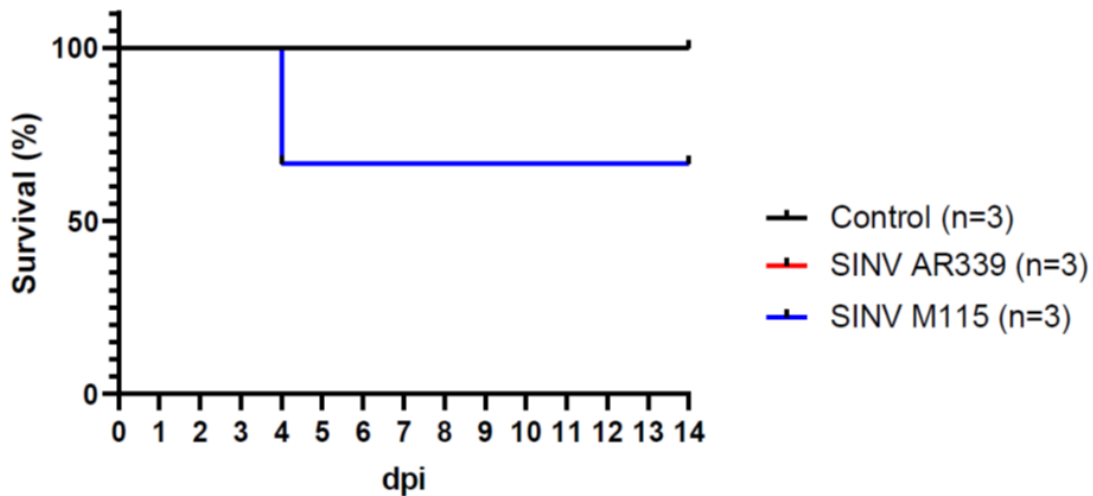
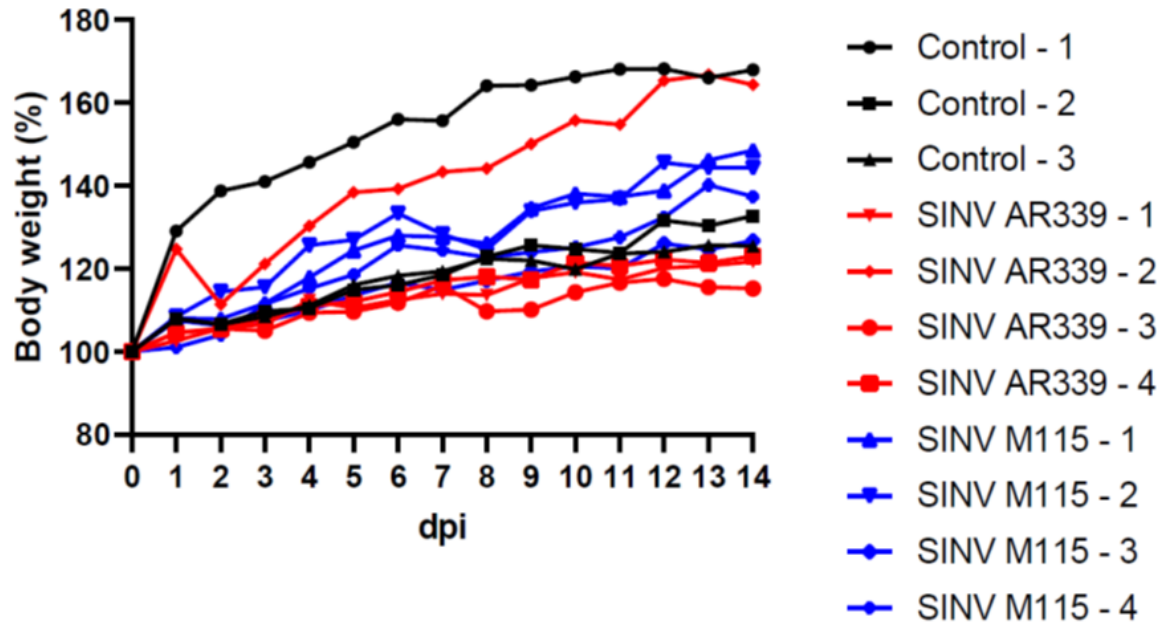


Fig. 5: Body weight change graph (A) and survival curve (B) of AG129 mice infected with 1 PFU of SINV

AG129 interferon knockout mice (n=3 per group) were inoculated SC into the nape of the neck with 50 μ L of 1 PFU of SINV M115 or AR339 and a control group was inoculated with MEM 2% FBS. Mice were monitored daily for body weight changes and survival up to 14 dpi. Percentage body weight change vs. time and Kaplan-Meier survival curves were plotted in Prism 9 (GraphPad software).

A

C57 BL/6 mice (1,000 PFU)



B

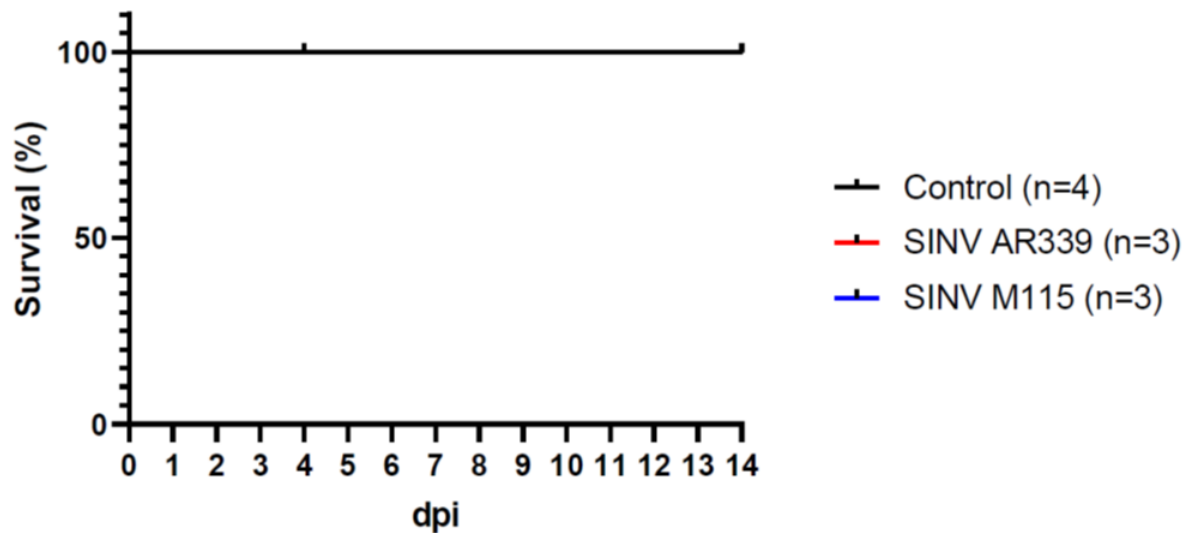


Fig. 6: Body weight change graph (A) and survival curve (B) of C57BL/6 mice infected with 1,000 PFU of SINV

C57BL/6 mice (n=4 per group) were inoculated SC into the nape of the neck with 1,000 PFU of SINV M115 or AR339. All mice were monitored daily for body weight changes and survival up to 14 dpi. Percentage body weight change vs. time and Kaplan-Meier survival curves were plotted in Prism 9 (GraphPad software).

Discussion

In this study, a new strain of SINV was successfully isolated from *Culex* mosquitoes captured in Zambia. The *in vitro* growth characteristics of SINV and its *in vivo* pathogenicity in mice correlated well with previously studied properties of this virus (19, 38). In type I/II IFN deficient mice, SINV has been shown to be fatal within 3 dpi and the disease progression is largely characterized by signs of viral hemorrhagic fever, inflammatory infiltrates in multiple organs and vascular leakage as well as reduced body weight as the infection proceeds (20). The Zambian SINV strain when inoculated into AG129 mice in this study produced mortality in all mice infected with 10 PFU titers within 3 dpi. These findings underscore the importance of the innate immune response mediated through interferon production as a mechanism to protect against SINV infection as has been demonstrated previously. Upon this premise it is evident that Zambian SINV M115 is highly pathogenic in an established immunodeficient animal model. Previous investigations have shown that some SINV strains, termed neuroadapted SINV (NSV), can cause pathogenesis in immunocompetent mice involving the central nervous system (CNS) through persistence of viral RNA in the CNS even after the virus has been cleared by antibody and T-cell responses, leading to the dissemination of the virus to the motor neurons of the musculoskeletal system (22, 40). The resulting pathology is characterized by age-dependent paralysis with neonatal mice being most severely affected (41). It has been suggested that amino acid changes in the envelope glycoproteins E1 and E2 are the determinants of neurovirulence in NSV compared with a low-passage AR339 strain of SINV (42). The C57BL/6 mice used in this study have been shown to be highly susceptible to the NSV strain (43); however, the Zambia SINV isolate, similar to AR339, did not exhibit pathogenicity in immunocompetent mice.

SINV has been demonstrated to exhibit cross-continental spread *via* major bird-migratory routes (13). The Zambian SINV strain discovered this time is assumed to circulate among birds and mosquitoes across Africa. However its significance in human cases in African countries remains unclear due to the limited genetic information available on human derived SINV. This study suggests that it is worthwhile to investigate how alphaviruses spread from a global epidemiological perspective. Mongu is located in the Western region of Zambia and shares its border with Angola. In previous reports surveying the risk of arbovirus introduction into Zambia, human travel to other nearby African nations of South Africa, Democratic Republic of Congo, and Angola were identified as contributors to the exposure of arboviruses, including Yellow Fever Virus, Zika Virus, and Dengue Virus (17, 44). In

addition to this, West Nile virus has been detected from mosquitoes captured in the Western Province of Zambia (15). Since SINV was detected from Mongu, the capital of Western Province, this suggests that this area may be a focus for arbovirus circulation and warrants further studies regarding surveillance and monitoring as a proactive measure for Zoonosis control efforts. Furthermore, this effort should take into account wildlife surveillance to record the cross-boundary movement of avian and mammalian intermediate hosts from Zambia to surrounding nations in order to estimate the risk of arbovirus introduction to and from Zambia. It has been suggested that a single introduction of SINV into an immunologically naïve population is enough to lead to the establishment of variants with epidemic potential (17, 45). SINV is known to cause an illness characterized by fever, rash, fatigue and muscle pain. It is therefore important to screen patients presenting to primary healthcare facilities with febrile illness for SINV as a part of the differential diagnosis.

Environmental assessments indicated that the North-western and Western Provinces of Zambia possess ample annual precipitation and humidity to facilitate the proliferation of mosquito species (44). Thus, the risk of mosquito-borne viruses being introduced and sustained in these regions through enzootic and epizootic cycles is relatively high. Other abiotic factors such as climate change now need to be considered which could directly impact bird migratory routes and mosquito distribution.

This study augments the current global knowledge on the distribution of Alphaviruses in general and SINV in particular as it has revealed a new locale where the virus was previously unknown to inhabit. Given the main biological mechanisms (migratory birds) by which the virus is spread and the fact that single introduction events of SINV to new locales in the past have led to establishment of enzootic and epidemic variants, this study provides the impetus for considering the potential threat of SINV spreading within Zambia and nearby territories within the African continent.

Chapter II: Isolation and characterization of Eilat virus from mosquitoes captured in Zambia

Summary

Eilat virus (EILV), an insect-specific alphaviruses, has been identified from two pools of *Culex* and *Anopheles* mosquitoes through alphavirus screening in mosquitoes captured in Zambia. EILV identified from *Culex quinquefasciatus* was successfully isolated using mosquito-derived C6/36 cells, and genetic characterization and proliferation in cultured cells were analyzed. The genetic characterization showed that Zambian EILV (strain zmq19_M44) exhibited a close phylogenetic relationship with other insect-specific alphaviruses and shared a close nucleotide identity to those of EILV isolate (90.4%) and Mwinilunga alphavirus (MWAV) (75.5%), which is a novel alphavirus previously identified in mosquitoes collected in Zambia. The EILV zmq19_M44 replicated in C6/36 cells and attained a saturating titer at 6 to 8 dpi but was unable to replicate in mammalian cell lines, Vero and BHK-21 cells. This study revealed that, in addition to MWAV and SINV, EILV is also present in mosquitoes inhabiting Zambia, indicating the existence of diverse alphaviruses in the region.

Introduction

EILV in the genus *Alphavirus* was first isolated from *Anopheles coustani* mosquitoes in Israel (23). It has subsequently only been detected in mosquitoes, *Culex pipiens* in Morocco, and isolated from *Culex univittatus* in Namibia. Insect-specific alphaviruses, including EILV have not been detected in humans or animals, and experimental studies using cultured cells have shown that its replication occurs exclusively in mosquito-derived cells (2, 26, 46). Five insect-specific alphavirus species have been identified to date and in the following countries, namely Eilat virus (Israel, Morocco, Namibia), Tai-Forest alphavirus (Côte d'Ivoire), Mwinilunga Alphavirus (Zambia), Agua Salud alphavirus (Panama) and Yada Yada virus (Australia) (2, 16, 23-25, 46-50). Of these insect-specific alphaviruses, EILV (2, 23, 25, 48) and Agua Salud alphavirus (47) have been successfully isolated. Thus, the number of insect-specific alphaviruses that have been isolated is limited, and the evolution of alphaviruses and the mechanisms of host specificity remain unknown. In this study, a new strain of EILV that was successfully isolated from mosquitoes was characterized.

Materials and Methods

Cells

C6/36 (CRL-1660, ATCC) and BHK-21 (RCB1423, RIKEN BRC) cells were maintained in EMEM containing 10% FBS under 5% CO₂ at 28 °C and 37 °C respectively. Vero cells (VERO C1008, CLR-1586) were maintained in DMEM containing 10% FBS under 5% CO₂ at 37 °C.

Mosquito collection and alphavirus screening

Mosquito surveillance was conducted in the Western part of Zambia (Mongu district) between 2017 and 2019 with permission from the Excellence in Research Ethics and Science (ERES) converge ethics committee (Ref. No. 1382-2020) (15).

In total, 5,843 female mosquitoes were captured and divided into 329 pools by species (maximum 40 mosquitoes per pool). The pooled mosquitoes were homogenized in EMEM with 2% FBS. A part of the homogenate was subjected to RNA extraction with Direct-zol RNA Miniprep kit (Zymo Research). Pan-alphavirus RT-PCR screenings were conducted as described in Chapter I. In brief, RT-PCR was performed with PrimeScript One Step RT-PCR Kit VER.2 (Takara Bio, Shiga, Japan) using pan-alpha primer sets, such as, nsP4-6692 F (5'-CAYACRYTRTTYGAYATGTCDGC-3') and pan-alpha nsP4-7152 R (5'-GCRTCDA TKATYTTBACYTCCAT-3') with the following conditions: 50 °C for 30 min, 94 °C for 2 min, 43 cycles of 94 °C for 30 sec, 52 °C for 30 sec, 72 °C for 30 sec, and 72 °C for 5 min 60 °C. The PCR products were visualized on agarose gels stained with ethidium bromide and purified on 2% (w/v) low melting SeaPlaque agarose (Lonza) and the MonoFas DNA Purification Kit I (GL Sciences). The sequence of the purified PCR products were analyzed by Sanger sequencing, and obtained sequences were analyzed by BLAST and described in Chapter I.

Virus isolation and whole genome sequencing

Either C6/36 or BHK-21 cells were inoculated with the homogenates positive for pan-Alpha virus RT-PCR and cultured for one week. After the second passage of the homogenate-inoculated cells, the supernatants from the cells showing a CPE were subjected

to cloning by limiting dilution methods using C6/36 cells. The lowest dilution of cell culture supernatant from CPE positive cells was inoculated into C6/36 cells. In brief, CPE-positive cell culture supernatant (50 μ L) was inoculated into C6/36 cells in 5 mL of MEM 2% FBS in a 10 mL cell culture dish and incubated at 28°C for 60 min. The inoculum was supplemented with a total of 10 mL of MEM 2% FBS and incubated at 28°C until CPE was observed. The supernatant containing virus was harvested and stored in 500 μ L aliquots at -80°C. The initial titer of this EILV stock was determined by the median tissue culture infectious dose (TCID₅₀) in C6/36 cells. For the TCID₅₀ assays, serially diluted supernatants were mixed with C6/36 cells at a concentration of 1.0×10^6 cells/mL in 96-well plates. Plates were incubated at 28 °C for 8 days and TCID₅₀ titer was determined by the Spearman-Kärber method.

The supernatants from cells infected with EILV were subjected to RNA extraction with Direct-zol RNA Miniprep kit. Whole genome sequencing of the purified RNA samples was conducted through the Illumina Miseq platform (Illumina) using Nextera XT DNA Library MiSeq Reagent Kit v3 (Illumina). The terminal sequence of the viral genome was determined by the 5'- and 3'- rapid amplification of cDNA ends (RACE using SMARTer RACE 5'/3' kit (Takara Bio) to obtain the terminal sequences as described in Chapter I.

qRT-PCR screening for EILV

qRT-PCR screening was performed on all the mosquito pools using the One Step TB Green PrimeScript PCR Kit II (Takara Bio) on a QuantStudio 3 real-time PCR system (ThermoFisher Scientific) with the following protocol: (42 °C for 5 min, 95 °C for 10 sec, 40 cycles of 95 °C for 3 sec 60 °C for 30 sec) using the primer sets as follows; zEilat 1067F (5'-CAACCATCTGCGACCAAATGA-3'), zEilat 1237R (5'-TGCTGAATCCTTGTGCTACGG-3'). The MIR was calculated by maximum likelihood estimation in the Pooled Infection Rate version 4.0 (36). The confidence interval (CI, 95%) was indicated in the range between the upper and lower bounds.

***In vitro* growth kinetics assay**

At the infection experiments, EILV titers were converted to PFU/mL by multiplying the TCID₅₀/mL values by 0.7 to calculate the MOI. For the growth kinetics assays, C6/36 cells in 6-well plates were infected with EILV at a MOI of 0.1. Similarly Vero cells and BHK

cells were inoculated with EILV at a MOI of 0.1 to assess the ability of EILV zmq19_M44 to infect mammalian cells and the infected mammalian cells were incubated at 37 °C for 8 days. Culture supernatants were harvested at different time points and subjected to titration by the TCID₅₀ assay.

Phylogenetic analysis

Genomic sequences of 40 reference strains of representative alphaviruses were obtained from GenBank. Multiple amino acid sequence alignment of coding regions of nonstructural and structural proteins were constructed with EILV_zmq19_M44 (accession number LC869669) and 40 other representative alphaviruses by MUSCLE in MEGA 11 software (37). Phylogenetic trees were constructed by the Maximum likelihood method using JTT model with 1,000 bootstrap replicates.

Results

Isolation of alphaviruses from *Zambian mosquitoes*

Alphavirus screening using pan-alphavirus RT-PCR detected two positive signals for a pool of *Culex quinquefasciatus* (zmq19_M44, 40 mosquitoes/pool) and a pool of *Anopheles coustani* (zmq19_M27, 30 mosquitoes/pool) captured in Mongu district, Western Zambia in 2019 (Table 1 in Chapter I, page 14). The sequence of both PCR products were identical and showed similarity to the nsP4 region of the EILV genome. EILV specific qRT-PCR was performed on all the mosquito pools collected in Mongu region to investigate EILV prevalence. It was shown that zmq19_M27 and zmq19_M44 were positive for EILV with an MIR of 1.51 (95% CI 0.09-7.02) in 538 *Anopheles* mosquitoes and 0.74 (95% CI: 0.04-3.55) in 871 *Culex* mosquitoes, respectively (Table 1 in Chapter I, page 14). Based on the results of the RT-PCR, the pool of zmq19_M44 was used for virus isolation using C6/36 cells. CPE in cells was observed after a second passage of cell supernatants inoculated with the mosquito lysate. The supernatants from the cells showing a CPE were subjected to isolate EILV by limiting dilution methods using C6/36 cells. The isolated virus was propagated further in C6/36 cells for the subsequent experiments.

Whole genome sequence and phylogenetic analysis of *Zambian EILV*

The whole genome sequence of the *Zambian EILV* (strain zmq19_M44, EILV M44) was determined using NGS and RACE analyses of RNA extracted from the supernatant of EILV-infected cells. The genome sequences (11,606 nt) of this virus has been submitted to the DDBJ/EMBL/GenBank databases under the accession number LC869669. EILV M44 showed 90.4% nucleotide sequence similarity to EILV EO329 (accession no. NC_018615), a strain isolated from *Anopheles coustani* mosquito in Israel (2) and also shares 75.5% nucleotide identity to MWAV previously identified from mosquitoes in Zambia (16) (Table 3).

Phylogenetic trees were constructed based on coding regions of nonstructural and structural proteins of representative Alphaviruses. *Zambian EILV* M44 formed a cluster with other EILV strains and segregated from other insect-specific alphaviruses such as MWAV and Tai Forest alphavirus in both the non-structural and structural proteins (Figs. 7A and B). Furthermore, the cluster containing EILV was phylogenetically closely related to the WEE

complex containing SINV. These results confirmed that the Zambian EILV M44 isolate is a new strain of EILV and resides in the phylogenetic cluster that is typical of insect-specific alphaviruses.

Growth kinetics of the isolated Zambian EILV

To investigate *in vitro* replication properties of the Zambian EILV M44, the growth kinetics of EILV M44 were examined in C6/36, Vero, and BHK cells. The growth of EILV M44 was exclusively observed in mosquito-derived C6/36 cells and attained a saturating titer of 10^7 TCID₅₀/mL at 144 hpi, while it failed to replicate in mammalian cells, Vero and BHK (Fig. 8). These growth property of EILV M44 were consistent with those of insect-specific alphaviruses that can replicate only in mosquito-derived cells (2).

Table 3

Nucleotide and Amino acid homology of EILV M44 to closely related alphaviruses

Sequence identity to Zambian Eilat virus zmq19_M44 (%)										
Strain	Nucleotide					Protein				
	Whole genome	nsP1	nsP2	nsP3	nsP4	Capsid	E3	E2	6k	E1
	11,606 nt	543 aa	809 aa	479 aa	608 aa	256 aa	63 aa	420 aa	55 aa	440 aa
Eilat virus isolate EO329	90.44	97.24	96.42	91.45	96.91	95.65	93.65	93.82	96.36	95.22
NC_018615.1										
Mwinilunga alphavirus Zmq16mw21	75.51	88.21	88.07	69.12	87.95	76.06	80.36	66.02	83.64	77.27
LC361437.1										

nt: nucleotides, aa: amino acids

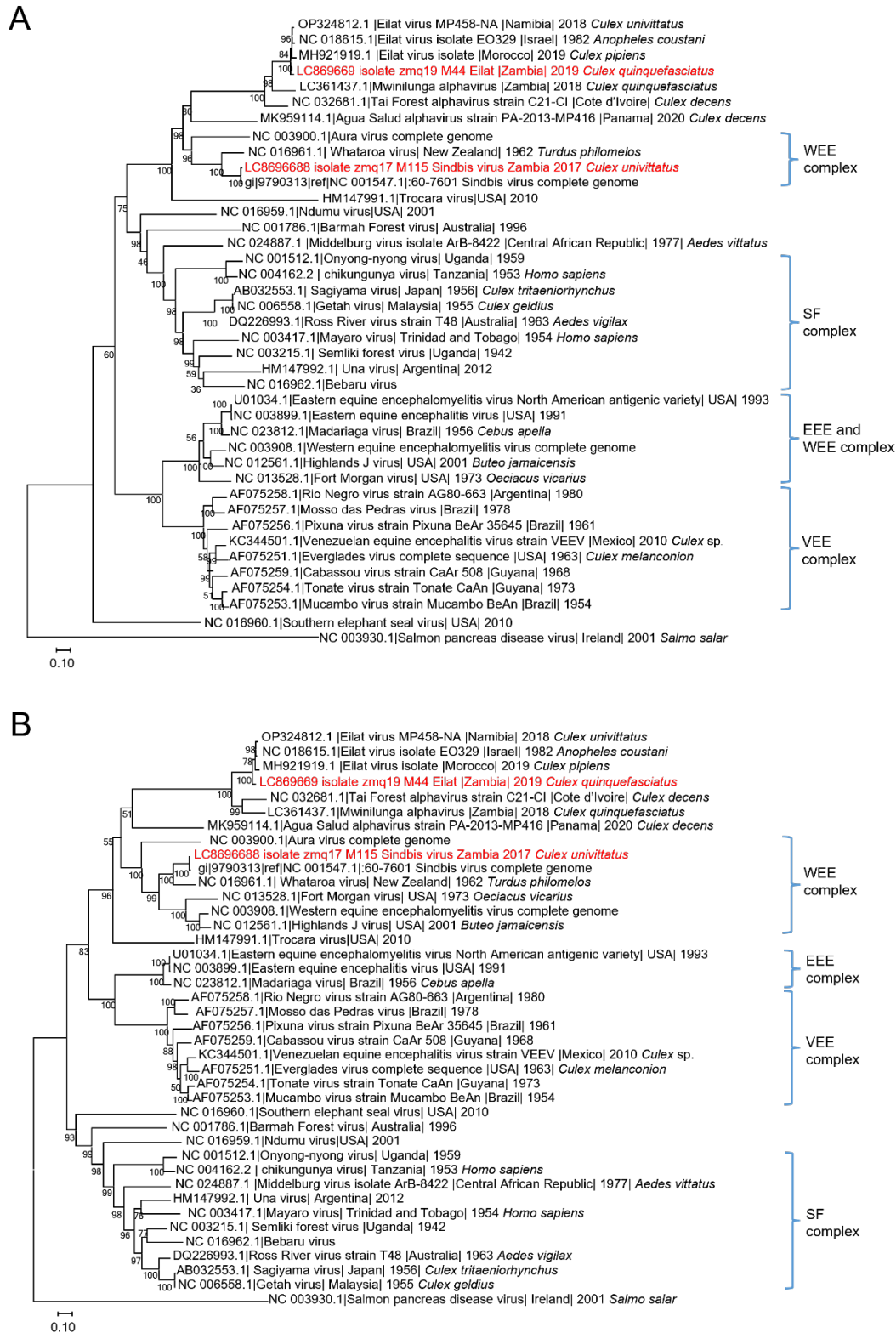


Fig. 7 Phylogenetic analysis based on amino acid coding regions of alphaviruses.

Phylogenetic analysis of non-structural proteins (A) and structural proteins (B) of alphaviruses. Phylogenetic trees were constructed by the Maximum Likelihood method using a JTT model with 1,000 bootstrap replicates. Red text indicates Zambian SIN V M115 and EILV M44 isolated in this study. Alphavirus serocomplexes are shown to the right side of the tree. The bootstrap values are shown at the branch node of each clade. The scale bars represent the number of substitutions per site.

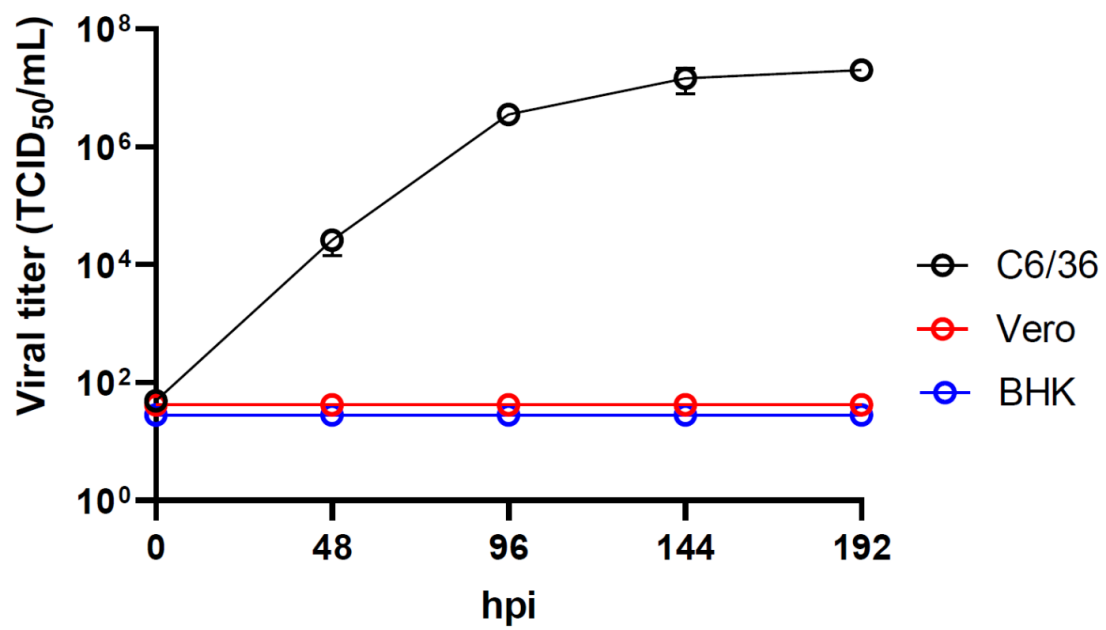


Fig 8: Growth kinetics of Zambian EILV M44.

Vero, BHK-21 and C6/36 cells were infected with EILV M44 at an MOI of 0.1. Titers at the indicated time points were determined by TCID₅₀. The values shown are mean $\pm$ SD of triplicate studies.

Discussion

The alphavirus screening of mosquitoes conducted in this study revealed the presence of EILV in Zambia. Growth kinetic analysis revealed that the isolated EILV M44 strain exclusively replicated in mosquito-derived cells, consistent with previous findings (2). Another suspected insect-specific alphavirus, MWAV, has also been found in *Culex* mosquitoes in the neighboring North-Western Province of Zambia (16). Known and unknown alphaviruses exhibit diversity and further research, including the possibility of unknown hosts other than mosquitoes for insect-specific alphaviruses, may promote elucidating the ecology and evolution of alphaviruses.

The transmission route of EILV into Zambia is puzzling due to the large geographical distance between Zambia, Israel, Morocco, and Namibia, and the limited opportunities for global dissemination of the virus. The EILV M44 strain is approximately 90% identical at the nucleotide level to other EILV strains, including those from neighboring Namibia, suggesting that EILV may circulate in a limited area and maintained independently in mosquitoes in Zambia. However, there remains the possibility that EILV could spread to other regions due to the presence of other unknown hosts or the movement of mosquitoes associated with the logistics of global trade.

General conclusion

The genus *Alphavirus* contains a diverse spectrum of viruses whose biological characteristics are not fully understood. Among them are SINV which infects mammals and birds, and EILV which is restricted to mosquitoes. This study represents the first report of simultaneous isolation of these two alphaviruses during an arbovirus survey in Zambia, providing important insights for arbovirus research, especially regarding biology and dynamics of alphaviruses. Although no human clinical cases have been reported in Zambia to date. This study suggested that SINV either circulates in Zambian mosquitoes or is seasonally introduced. SINV infection may have contributed to some previously reported undiagnosed febrile illness in Zambia. Further serological and genetic surveillance of alphaviruses are needed to determine the prevalence of SINV infection in humans and animals in Zambia.

Notably, the only isolation of EILV in Zambia represents the fourth successful isolation worldwide. Given that EILV is known to inhabit mosquitoes, how it spreads internationally remains an intriguing question. One possibility is logistics routes associated with global trade, where infected mosquitoes could be mixed into shipping cargo and transported long distances from their original regions to new regions.

As described above this study will contribute to public health measures against alphaviral infections in Zambia and provides insights on future research on emerging arbovirus-related zoonotic diseases for Zoonosis Control efforts related to emerging arboviruses.

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