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Australian isolates of cell fusing agent virus and Culex flavivirus provide new tools to study insect-specific flaviviruses

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Abstract

Cell fusing agent virus (CFAV) and *Culex* Flavivirus virus (CxFV) represent the prototype insect-specific flaviviruses (ISFs) found in *Aedes* and *Culex* species, respectively. While these viruses have been isolated from mosquitoes on several different continents, no isolates have been reported from Australian mosquitoes. In this study we successfully isolated CFAV from *Aedes aegypti* originating from Cairns in North Queensland, Australia and CxFV from *Culex quinquefasciatus* collected from Brisbane in South-East Queensland, representing the first field isolates for both viruses from Australia. Phylogenetic analysis of these isolates revealed that the new CFAV isolates were closely related to CFAV sequences previously detected by deep sequencing of pools of *Aedes aegypti* collected in North Queensland, while the genome sequences of the Brisbane CxFV isolates were most closely related to isolates of CxFV from *Culex quinquefasciatus* collected from Mexico in 2008. Using these Australian isolates, we report the first infectious clone created for CFAV, and the first infectious clone of the Australian strain of CxFV. Growth kinetics revealed that infectious clones of CFAV and CxFV replicated more efficiently than their wild-type counterparts, reaching titres more than 10^8 $TCID_{50}$/ml. Deep-sequencing of these isolates indicated that differences in replication were due to co-infections of mosquito-derived native viruses with other viruses absent in infectious clone stocks. Using a panel of novel monoclonal antibodies raised against the CFAV virion proteins, we demonstrated antigenic relatedness between CFAV and CxFV and other lineage I ISFs, particularly those carrying the conserved FKW motif at positions 106–108 in the fusion loop region of the viral envelope protein. All generated mAbs bound CFAV with one mAb additionally recognising the ISFs CxFV, Palm Creek virus (PCV), Parramatta River virus (PaRV) and Niénokoué virus (NIEV). While these mAbs did not bind CxFV and CFAV proteins in mosquitoes injected with the infectious clones, the first immunofluorescence analysis of disseminated of CFAV in *Aedes* mosquitoes was visualised through anti-dsRNA staining, showing widespread infection across multiple mosquito tissues, including the midgut and ovaries. These advancements in the development of elementary tools provide opportunity to further our understanding of ISFs and pave the way for their potential application in laboratory research, as well as innovative mosquito-borne disease control strategies.

Introduction

The genus *Orthoflavivirus* (family: *Flaviviridae*) contains approximately 60 viruses that can be broadly divided into vector-borne viruses and those with no known vector[1]. The genus includes several important human pathogens, such as yellow fever (YFV), Japanese encephalitis (JEV), dengue (DENV), Zika (ZIKV) and West Nile (WNV) viruses. More recently, a subgroup of orthoflaviviruses restricted to replication in arthropod hosts only, termed insect-specific flaviviruses (ISFs), have been identified[2]. ISFs have a global distribution and infect multiple mosquito genera. Beyond providing insight into orthoflavivirus evolution, accumulating evidence suggests that ISFs may influence the transmission of vertebrate-infecting orthoflaviviruses, such as WNV and ZIKV, in co-infected mosquitoes[3,4]. ISFs have also been exploited to generate chimeric viruses expressing the structural proteins of pathogenic orthoflavivirus for applications in vaccine and diagnostic development and to investigate the molecular basis for host restriction[5–11].

The first ISF, cell fusing agent virus (CFAV), was identified in 1975 as the cause of cytopathic effects in an *Ae. aegypti*-derived cell line[12]. Although CFAV was not isolated from wild mosquitoes until 2006 in Puerto Rico, the closely related Kamiti River virus was detected and isolated from *Ae. macintoshi* mosquitoes captured in Kenya in 1999[13]. Since then, more than an additional 30 ISFs have been detected or isolated from mosquito species worldwide and classified into two distinct groups; classical ISFs and dual-host affiliated ISFs. Classical ISFs are phenotypically distinct from all other known orthoflaviviruses and are often thought to represent ancestral forms from which vertebrate-infecting flaviviruses (VIFs) have evolved[14]. In contrast, dual-host affiliated ISFs are not monophyletic and cluster more closely to VIFs despite retaining an insect-specific phenotype[15,16]. Within both lineages, viruses also cluster according to their mosquito host genera, with *Aedes, Culex*, or *Anopheles*-associated ISFs forming distinct phylogenetic groups[14].

Among the classical ISFs, CFAV and *Culex* flavivirus (CxFV) are the most widely distributed species. CFAV has been isolated from *Aedes* populations across every continent except Antarctica[17–24], while CxFV has been reported extensively from *Culex* mosquitoes worldwide[25–28]. Despite previous metagenomic detection of CFAV RNA in *Ae. aegypti* from Northern Queensland[29], the virus has not been isolated from mosquitoes trapped on this continent. Similarly, CxFV has not been detected, despite extensive screening of Australian mosquito collections. The availability of experimental tools to investigate these viruses has also remained limited.

Infectious clones have become indispensable tools for studying orthoflavivirus replication[30,31], virion structure[6,32], host interactions[11,33,34] and mechanisms of transmission[35,36]. For ISFs, infectious clones also enable mechanistic studies of host restriction and the development of novel biotechnology applications while avoiding confounding effects of co-infecting viruses commonly present in field isolates[37]. Likewise, monoclonal antibodies (mAbs) are essential reagents for defining virus antigenicity, mapping epitopes, assessing localisation of virus within tissues and detecting infection[38]. Together, these complementary tools provide a powerful framework for investigating ISF biology.

Here, we report the first isolations of CFAV and CxFV from Australian mosquitoes and describe their genetic and antigenic characteristics. We established the first reported infectious clone of CFAV and recovered a CxFV infectious clone using circular polymerase extension reaction (CPER), together with the generation of virus-specific mAbs. These resources substantially expand the experimental toolkit available for ISFs and provide new platforms for investigating their biology and biotechnological applications.

Materials and Methods

Ethics statement and approvals

All mouse work was conducted in accordance with the "Australian Code for the Care and Use of Animals for Scientific Purposes" as defined by the National Health and Medical Research Council of Australia. The animal experiments were approved by the University of Queensland Animal Ethics Committee (AEC permit number 2023/AE000424). All work with recombinantly-generated viruses was approved by the UQ Institutional Biosafety Committee (UQ IBC approval number IBC/1505/SCMB/2023).

Mosquito Samples

Aedes aegypti Cairns and Innisfail strain mosquitoes used in this study were obtained from colonies established from eggs collected from *Wolbachia*-free locations in Cairns in 2014 and Innisfail in 2016 (both in Queensland, Australia), respectively. The strains were subsequently maintained in the QIMR Berghofer insectary (27 ^{o}C, 75% relative humidity, 12:12 hr day night cycle with dawn dusk fading). Larvae were fed on TetraMin tropical fish food flakes and adult mosquitoes were maintained in 30 x 30 x 30 cm cages (BugDorm, MegaView Science Education Services Co., Ltd., Taiwan) and provided with 10% sugar solution *ad libitum* and weekly blood meals of defibrinated sheep blood for egg production.

Culex quinquefasciatus mosquitoes assessed in this study were captured in a Brisbane backyard (Queensland, Australia using BG sentinel traps set overnight without a lure or CO_2.

Mosquito processing

Whole mosquitoes were separated into pools containing between 1–20 adult mosquitoes (Table S1). These were mixed with 1–3 sterile beads and between 500–900 µl of RPMI containing 2% FBS were added and the samples were homogenised at 30 Hz for 4 min (Tissuelyser II, Qiagen). Homogenates were clarified by centrifuging at 11, 000 xg for 5 min, before being filtered through a 0.22 µM filter, and inoculated onto C6/36 monolayers, as described below.

Screening of mosquito homogenate by the MAVRIC ELISA system.

The filtered mosquito homogenate was screened through the detection of double-stranded RNA (dsRNA; monoclonal antibodies against viral RNA intermediates in Cells, MAVRIC) as previously described[39,40]. Briefly, mosquito homogenate was inoculated onto confluent C6/36 cell monolayers in 96-well microplates and incubated at 28 ^{o}C for 5 dpi. Following this, the supernatant was harvested and stored at -80 ^{o}C prior to fixing the cell monolayers with an ELISA fixative buffer containing 4% (v/v) formaldehyde and 0.5% Triton-X 100 in PBS. Enzyme Linked Immunosorbant Assay (ELISA) was performed using anti-dsRNA mAbs 3G1 and 2G4[39]. Briefly, plates that had been blocked with ELISA blocking buffer (0.05M Tris/HCl (pH 8.0), 1 mM EDTA, 0.15 M NaCl, 0.05% (v/v) Tween-20, 0.2% (w/v) casein) were probed with a pre-determined dilution of mAbs 3G1 and 2G4 and incubated at 37 ^{o}C for 1 hr. Following this, the plates were washed four times with PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% (v/v) Tween-20, pH 6.74) and probed with horse-radish peroxidase-conjugated goat anti-mouse Ig H + L (DAKO, diluted 1/2000) and further incubated for 1 hr before washing an additional six times with PBST. Finally, substrate solution [1 mM 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 3 mM H_2O_2 in a buffer prepared by mixing 0.1M citric acid with 0.2 Na_2HPO_4 to give a pH of 4.2] was added and plates were incubated in the dark and at room temperature for 1 hr. Absorbance was measured at 405 nm. Samples were considered positive for dsRNA or +ssRNA viruses if the optical densities (ODs) were greater than twice the average of the mock-infected wells.

RNA extraction and RT-PCR Screening

RNA extraction and RT-PCR Screening
Mosquito homogenate samples that tested positive for dsRNA via MAVRIC were further analysed by RT-PCR. Viral RNA was extracted using a Nucleospin Viral RNA Isolation kit (Macherey-Nagel, Düren, Germany) as per manufacturer's instructions and the RNA was eluted in RNase-free water and stored at -80 ^{o}C. RT-PCR was performed using an Invitrogen Superscript III One-step RT-PCR system with Platinum *Taq* polymerase using the pan-flavivirus primer set FU2 and cFD3[41] or the pan-ISF primer set ISF_F1 (GGGCAAGTARBMACTTATGCVTTGAACAC) and ISF_R1 (GCCCACATCTGGGCRTRNGCCTTNGC)[42] as per the manufacturer's instructions. Amplicons of the expected size were purified by agarose gel electrophoresis and a Gel Extract II PCR Clean-up kit (Macherey-Nagel) as per the manufacturer's instructions before Sanger sequencing was performed by the Australian Genome Research Facility (AGRF, Brisbane, Australia).

Deep sequencing and phylogenetic analyses

Next generation sequencing was performed to obtain the genomes of new virus isolates identified in this study. Genomic sequence of these viruses was obtained by extracting RNA from virions using Tri-reagent LS (ThermoFisher Scientific) as per the manufacturer's instructions. The purified RNA was sequenced at the AGRF using the NovaSeq SP Illumina platform, generating 2x150- base paired reads. The viral genome was assembled using the closest relative (as determined by the RT-PCR screening) as a reference genome and Geneious Prime software (version 2023.1.1) and the data deposited under Genbank numbers PZ988475 (CFAV) and PZ988476 (CxFV).

To resolve the untranslated regions (UTRs) of orthoflavivirus sequences detected, the genome was circularisation as previously described[10,43]. Briefly, 1 µg of RNA from concentrated CFAV-positive or CxFV-positive culture supernatant was extracted using TRIzol reagent (Thermofisher Scientific, Massachusetts, United States) as per the manufacturer's instructions. The viral RNA was then decapped with tobacco acid pyrophosphatase at 37°C for 1 hr and circularised overnight at 4°C using T4 RNA ligase. The circularised RNA was then converted into cDNA using Superscript IV (Thermofisher Scientific) with a virus-specific reverse primer targeting the prM gene (Table S2). The junction between the 5' and 3' UTRs (Table S2) was amplified via PCR using Phusion High-Fidelity DNA Polymerase as per the manufacturer's instructions (New England Biolabs, NEB) and sequenced by Sanger sequencing (AGRF). The junction site of the UTRs was identified through alignment with orthoflavivirus consensus sequences.

Amino acid multiple sequence alignments of the entire open reading frame (ORF) were performed with MAFFT v1.3.7 with the algorithm selected automatically, a scoring matrix of 200PAMk = 2, a gap open penalty of 1.53 and an offset value of 0.123. FastTree v1.0 was then used to construct phylogenetic trees using the maximum likelihood approximation method with a general time-reversible model, optimised for gamma20 likelihood. The branch support values were calculated using a Shimodaira-Hasegawa test[44].

Cell and virus culture

C6/36 (*Aedes albopictus)* cells were maintained at 28 ^{o}C in Rosewell Park Memorial Institute 1640 (RPMI) media and supplemented with 5% heat-inactivated foetal bovine serum (FBS) and 50 U/mL penicillin, 50 µg/mL streptomycin and 2 mmol/L L-glutamine.

The viruses used herein were: Binjari virus (BinJV; Genbank MG587038), Palm Creek virus (PCV, Genbank KC505248), Parramatta River virus (PaRV, Genbank KT192549) and West Nile virus, Kunjin subtype (WNV_{KUN}), the latter an infectious clone (FLSDX) derived from the MRM61C prototype isolate (Genbank AY274504.1). Virus stocks were generated by infecting C6/36 cell monolayers with virus at a multiplicity of infection (MOI) of 0.1, or a 1/100 dilution if the titre of the virus was unknown. After incubation with rocking at 28 ^{o}C for 1 hr, the inoculum was removed and replaced with fresh growth media containing 2% FBS. Supernatant was harvested when extensive cytopathic effect (CPE) was observed or 5 dpi, which ever occurred first, by centrifuging at 3,000 rpm (Jouan CR132 centrifuge) and 4 ^{o}C for 10 min. The clarified supernatant was supplemented with additional FBS to increase the total concentration to 10% and stored at -80 ^{o}C until required. The virus stock titres were calculated based on the 50% tissue culture infectious dose ($TCID_{50}$)[45] using a cocktail of the anti-dsRNA monoclonal antibodies (mAbs) 2G4 and 3G1[39].

Development of infectious clones

Infectious clones of both CFAV (pool ID: Cairns wild-type male, Table S1) and CxFV (pool ID: HS002, Table S1) were created by circular polymerase extension reaction (CPER) technique as previously described [46]. Briefly, CFAV and CxFV viral RNA was converted to cDNA using SuperScript IV Reverse Transcriptase (Thermofisher Scientific) and random primers or a gene-specific virus-specific reverse primer as per the manufacturer's instructions. The cDNAs were then used as templates for a set of primer pairs (Table S2) to produce five dsDNA fragments spanning the CFAV and CxFV genomes with overlapping 5' and 3' ends. A silent mutation in the NS4B/NS5 junction for each virus was included to create a genetic signature for the infectious clone (Table S2). The linker sequence containing the AePUb promoter, SV40 pA and HDVr was amplified from plasmid templates as previously described[47]. The reverse primers at the 3' end of the AePUb promoter was designed to contain a hammerhead ribozyme (HH Rbz) sequence prior to the ISF 5' UTR to ensure precise cleavage of the viral genome 5' terminus. For each CPER assembly, 0.1 pmol of each viral cDNA fragment was added to a Q5 High-Fidelity DNA Polymerase (New England Biolabs, NEB) PCR as per the manufacturer's instructions. Thermal cycling conditions were as follows: [98 ^{o}C, 5 min]$_{x1}$, [98 ^{o}C, 30 sec; 57 ^{o}C, 30 sec; 72 ^{o}C, 6 min]$_{x2}$, [98 ^{o}C, 30 sec; 57 ^{o}C, 30 sec; 72 ^{o}C, 8 min]$_{x10}$. The entire CPER was then transfected into C6/36 cell monolayers using TransIT-LT1 Transfection Reagent (Mirus Bio, Madison, United States) as per the manufacturer's instructions, and the passage 0 (P_0) cell culture supernatants were harvested six days post-transfection and stored at -80 ^{o}C.

To confirm the presence of replicating virus following CPER, preliminary analysis was performed by trypsinising P_0 C6/36 monolayers and seeding the cells onto glass coverslips. The cells were incubated for a further 24 hr prior to being fixed in ice-cold 100% acetone. Immunofluorescence assays (IFA) were then performed as described below, using anti-dsRNA mAbs 2G4[6,10]. The infectious clones were deep sequenced as described above to confirm the successful recovery of the infectious clone and absence of contaminating wild-type virus.

Production of chimeras for antigenic analysis and mAb production

For antigenic analyses using mAbs (described below), four BinJV-based chimeras expressing the prM and E genes of ISFs were produced using the same strategy as for other BinJ/ISF-prME chimeras previously published[10]. Overlapping synthetic dsDNA prM/E inserts for the following viruses were used: CFAV (NC_001564; bCFAV), Nienokoue virus (NIEV, JQ957875; bNIEV), Kamiti river virus (KRV, NC_005064, bKRV) and Nakiwogo virus (NAKV, NC_030400, bNAKV) (Table S3). Recovery of replicating virus was confirmed by IFA using pan-flavivirus NS1 mAb 4G4[6,10,48] (described below) and sequencing over the junctions of the prME insert to confirm chimera identity.

mAb Production

To generate mAbs that were specific to CFAV, bCFAV virions were concentrated and purified using methods previously published[32]. Briefly, C6/36 monolayers were infected with bCFAV at an MOI of 0.1. Supernatant was collected at 3-, 5-, 7- and 9-dpi. The virus culture supernatant was clarified by centrifugation at 3000 xg for 30min at 4 ^{o}C and filtered through a 0.22 µM filter. After each collection, cells were replenished with fresh RPMI containing 2% FBS. The virions were precipitated from the harvested culture supernatant via addition of polyethylene glycol 8000 to a final concentration of 8% and slow stirring overnight at 4 ^{o}C. The virus was pelleted at 8,000 rpm (Beckman Coulter JLA 10.500 rotor) for 1.5 hr at 4 ^{o}C, before ultracentrifugation through a sucrose cushion and potassium tartrate gradient as previously described[6,49]. Purified virus was collected and buffer exchanged into sterile PBS using a 30 kDA molecular weight cut-off Amicon filter, before quantifying the total protein concentration using a Nanodrop One Spectrophotometer (ThermoFisher) by measuring absorbance at 280 nm (A280) and storing at 4 ^{o}C. Following virus purification and quantification, BALB/c mice (Ozgene, Murdoch, Western Australia) were immunised twice via the subcutaneous route with purified bCFAV virions, along with the inulin-based adjuvant Advax (Vaxine Ltd, Adelaide, Australia). The mice were further boosted with purified bCFAV virions by intravenous injection 4 days prior to harvesting of the spleen. Fusion of spleen cells with NSO myeloma cells (European Collection of Cell Cultures) was performed as previously described[32,50,51]. Hybridomas secreting CFAV- and CxFV-reactive antibodies were identified by fixed-cell ELISA as previously described[32,50,51]. The mAbs were then further analysed for reactivity using Western blot and fixed-cell ELISAs for cross-reactivity to other orthoflaviviruses (described below), with incubation steps being performed at 37 ^{o}C as previously described[52]. The isotype of each mAb was determined using mouse mAb isotyping reagents (Sigma-Aldrich), according to the manufacturer's instructions.

Western Blot

Viral protein expression was analysed by Western blot using lysates prepared from C6/36 cells infected with the CFAV infectious clone. At 7 dpi, infected C6/36 monolayers were washed twice with sterile PBS and lysed directly into BS9 buffer (120 mM NaCl, 50 mM H_3BO_3, 1% Triton X-100 and 0,1% SDS, pH 9.0) supplemented with protease inhibitors (Sigma Aldrich P8340, 1:100 dilution). Flasks were frozen at -80°C overnight with lysis buffer to enhance release of viral proteins. Supernatants were aliquoted and stored at -80°C until use. Uninfected C6/36 cell monolayers were also harvested via lysis to produce mock lysate.

CFAV-infected cell lysate (10 µL/well) was mixed with 4× LDS sample buffer, incubated at 95°C for 5 min, and resolved on 4–12% Bis-Tris or 4–20% Tris-Glycine SDS-polyacrylamide gels (Invitrogen). For reduction, the samples were also mixed with DTT. The resolved proteins were then transferred to nitrocellulose blotting membranes at 30V for 1 hr, before blocking in ELISA blocking buffer overnight at 4 ^{o}C. Following this, the mAbs as hybridoma supernatant were diluted 1:10 in ELISA blocking buffer and incubated at room temperature for 1 hr with rocking. After three washes with Tris-buffered saline containing Tween-20 (TBST; 150 mM NaCl, 20 mM Tris, 1% (v/v) Tween-20, pH 7.5), membranes were incubated with goat anti mouse Ig HRP-conjugated secondary antibody (IRDye 800CW, LI-COR 1:5,000) for 1 hr at room temperature with rocking, before washing an additional 3 times with TBST and imaging with an Odyssey CLx scanner (LI-COR).

Negative staining electron microscopy

Concentrated and purified CFAV infectious clone virions were adsorbed onto carbon-coated grids (ProSciTech, Thuringowa, QLD, Australia) for 2 min and glow-discharged for 5 sec in 25 mA. The grids were blotted and washed three times in water and stained twice with a 2% uranyl acetate solution, with blotting in between. The grids were air-dried and imaged using a Hitachi HT7700 microscope operated at 120 Kv.

Immunofluorescence Assay (IFA)

Indirect IFAs were performed as previously described[6,10]. Cell monolayers that had been fixed onto glass coverslips using 100% ice-cold acetone, were blocked with ELISA blocking buffer for 30–60 minutes on a rocker before being probed with pre-diluted mAb as hybridoma supernatant and incubated for an additional 1 hr with rocking. After three washes with PBST, secondary antibody (Alex Fluor goat anti-mouse Ig 488, 1:700 dilution) was applied for 1 hr in darkness. Following another set of washes, Hoechst 33342 stain (Thermofisher Scientific, 1:700 dilution) was added and incubated for 5 min in darkness. After washing with PBST, the coverslips were mounted onto glass slides using Prolong Gold antifade reagent (Thermofisher Scientific) and allowed to dry in the dark until imaging on a Zeiss LSM900 confocal microscope.

Antigenic analyses

To assess the antigenic relationships of the new isolates of CFAV and CxFV with other ISFs and VIFs, C6/36 cell monolayers grown in 96-well plates were infected with the CFAV and CxFV infectious clones, as well as a panel of reference viruses (Classical ISFs PaRV, PCV, bKRV, bNIEV and bNAKV and dual-host affiliated ISFs BinJV, bNHUV[10] and bILOV[10], as well as the vertebrate-infecting orthoflavivirus (VIF) WNV_{KUN}). The reactivity of a suite of mAbs directed to the structural proteins (prM and E) of each of the viruses was then assessed by fixed-cell ELISA as described above, titrating the supernatant from neat in 2-fold dilutions in triplicate.

Comparative Growth Analysis

Growth characteristics of infectious clones and mosquito-derived isolates were compared in C6/36 cells. Viral stocks at passage three were inoculated in triplicate at a MOI of 0.1 and incubated for 6 dpi. Viral titres were quantified as $TCID_{50}$/mL, and statistical significance between wild-type isolates (WT) and infectious clones (IC) was determined using an unpaired Welch's *t*-test.

Mosquito infection with CFAV infectious clone

Female *Aedes aegypti* mosquitoes (Innisfail strain) were reared under standard insectary conditions (12:12 day:night, 28°C and ~ 70% humidity, data not shown). Approximately 40 mosquitoes were included per experimental group. Mosquitoes were CO_2-anaesthetised and placed on a chilled surface, then intrathoracically inoculated with 138 nL of $CFAV_{ic}$ viral supernatant (derived from infected C6/36 cells) by intrathoracic microinjection (ITM). Injections were performed using a Nanoject II microinjector (Drummond Scientific) with heat-drawn borosilicate glass capillary needles. Following inoculation, mosquitoes were transferred to recovery cups and maintained at 28°C in the insectary with access to 10% sucrose ad libitum for 8 days. Following, to detect $CFAV_{ic}$ replication within mosquito tissues, the legs and wings were removed before fixing the whole body in 4% paraformaldehyde at 4 ^{o}C overnight. Subsequently, the mosquito bodies were transferred to 70% ethanol before being processed for paraffin embedding by the QIMR Histotechnology Facility using standard procedures. The tissues were dehydrated through an increasing graded ethanol series, cleared in xylene and embedded in paraffin wax prior to sectioning.

For the IFA staining, the paraffin-embedded mosquito sections were dewaxed and rehydrated through xylene and a descending ethanol series to water using a Leica Autostainer. Sections were treated with 2% hydrogen peroxide in Tris-buffered saline (TBS) for 10 min at room temperature to quench endogenous peroxidase activity, followed by blocking of endogenous mouse immunoglobulins with donkey anti-mouse Fab fragments (Jackson ImmunoResearch; 1:50 in Rodent Block M; 60 min) and non-specific antibody binding with Background Sniper supplemented with 2% bovine serum albumin (BSA; 15 min), all at RT. Sections were incubated overnight at 4°C in a humidified chamber with primary antibody (2G4, CF3-2D5 or CF3-3D1) diluted 1:200 in Da Vinci Green antibody diluent. Following this, sections were subsequently incubated with Alexa Fluor 555-conjugated anti-mouse IgG secondary antibody (1:300 in TBS) for 1 hr at room temperature in the dark, counterstained with 4',6-diamidino-2-phenylindole (DAPI) for 3 min, and mounted using Dako fluorescent mounting medium or an equivalent antifade medium.

Statistical analyses

Statistical analyses were performed in GraphPad Prism version 10.5.0 for macOS (GraphPad Software, Boston, MA, USA; www.graphpad.com). Statistical analyses for antigenic assessment were performed results using non-linear regression with one-site specific binding (Graphpad Prism, version 10).

Results

Detection and isolation of CFAV in Ae. aegypti mosquitoes from a laboratory colony sourced from Cairns.

Following reports of ISVs often being detected in colony mosquitoes[29,53], *Ae. aegypti* initially captured in Cairns or Innisfail in North Queensland, Australia and maintained in colonies at the Queensland Institute of Medical Research Berghofer (QIMR-B, Cairns colony, Table 1) were tested for the presence of ISVs through viral isolation. The colonies were established from eggs collected outside *Wolbachia* release zones in 2015 and 2016, respectively, and previously found to be *Wolbachia* negative by PCR. Of the 16 pools tested, two pools of *Ae. aegypti* Cairns adults tested positive for virus when inoculated C6/36 cell monolayers were probed for replicative viral dsRNA by MAVRIC ELISA[39]. Subsequent RT-PCR analysis using pan-ISF primers and Sanger sequencing identified the presence of CFAV, with each isolate sharing a 98% nucleotide identity over a 395 bp region of the NS5 gene to CFAV. Further analysis indicated that this sequence was consistent with exogenous viral RNA and was not attributable to a similar endogenous orthoflaviviral element which have previously been reported in the *Ae. aegypti* genome[54] (Table S4). No other *Ae. aegypti* pools captured from other locations tested positive for dsRNA by MAVRIC-ELISA, despite several passages on mosquito cells being performed prior to testing (Table S1). These data represent the first reported natural isolates of CFAV from an Australian mosquito population.

Table 1
Summary of Aedes aegypti and Culex quinquefasciatus pools screened by MAVRIC.

Mosquito Species	Year of Collection	Number of screened pools	Number of MAVRIC positive pools	Orthoflavivirus positive pools	Virus identity
Cairns, Queensland					
Aedes aegypti	2015	4	2	2	CFAV
Innisfail, Queensland					
Aedes aegypti	2016	2	0	0	N/A
Brisbane, Queensland					
Culex quinquefasciatus	2020	47	24	21	CxFV

Deep sequencing was performed on a single isolate (Cairns wild-type (WT) male) to elucidate the full ORF. The isolate shares a 99.9% nucleotide and 100% amino acid identity with a CFAV sequence detected in metagenomics studies of *Ae. aegypti* mosquitoes captured in a similar location in Cairns, from which an infectious isolate was not obtained[29]. Phylogenetically, the two Cairns sequences group closely with sequences detected in *Ae. aegypti* mosquitoes captured in Southeast Asia, including Thailand (Fig. 1, Table S5).

Figure 1: Phylogenetic analysis of available entire ORFs of CFAV available in GenBank including the study isolate. CFAV amino acid sequences where the complete ORF was available were aligned using MAFFT (Scoring matrix: BLOSUM62; Gap open penalty: 1.53; Offset value: 0.123), before

constructing a phylogenetic tree using FastTree, Whelan-And-Goldman model. Kamiti River virus (KRV, accession number AY49905) was used as an outgroup. #prototype sequence; *colony-derived isolate.

Detection and isolation of CxFV from Culex quinquefasciatus collected in Brisbane, Australia

Cx. quinquefasciatus mosquitoes were trapped from a private backyard in the Brisbane suburb of Graceville in late 2020 and pools of mosquitoes were tested (n = 47 pools, 10 mosquitoes/pool) for the presence of ISVs. Of these, 24/47 pools tested positive by MAVRIC ELISA, and 21/24 of the MAVRIC-positive samples yielded RT-PCR products of the expected size of 845 bp for orthoflavivirus NS5 sequence using the pan-orthoflavivirus-specific primers Fu2 and cFD3[41]. Partial sequencing of the NS5 gene revealed that the viral RNA from these pools shared a 99% nucleotide identity to each other, and a 95% nucleotide identity to CxFV. Completion of the entire ORF by deep sequencing confirmed that this isolate (HS045) was CxFV and shares a 97.6% nucleotide and 99.3% amino acid similarity to a strain obtained from *Cx. vishnui* mosquitoes captured in Cambodia (Cv21 strain, accession number OR479692) (Fig. 2, Table S6). To the best of our knowledge these represent the first isolates of CxFV from Australian mosquitoes.

Detection of other viral sequences in Australian mosquitoes

Three *Cx. quinquefasciatus* pools were MAVRIC-positive and orthoflavivirus-negative by pan-orthoflavivirus RT-PCR and also tested negative for the presence of several other viruses that are commonly isolated in Australian mosquitoes, including Liao ning virus (reovirus)[55] and Castlerea virus (negevirus)[56] using virus-specific RT-PCRs (data not shown). Deep sequencing analysis of representative pools HS003 and HS042 detected two distinct complete viral genomes that clustered closely with those of Culex iflavivirus-like 1 (CILV1) and Culex iflavirus-like 4 (CILV4) (Table 3), previously reported from metagenomic sequencing of *Culex* species captured in California, United States of America[57] (Table S1). While CILV1 has previously been detected, but not isolated, in *Cx. pipiens* mosquitoes captured in Perth, Australia[58], CILV4 has not. While these two sequences were identified in Brisbane *Cx. quinquefasciatus* samples that were positive for replicating dsRNA in inoculated cells but tested negative for orthoflavivirus RNA by RT-PCR, and were the only viral sequences detected with high level reads in their respective pools, we have likely obtained the first viable isolates of CILV1 and CILV4. Deep sequencing was also performed on a pool of the *Ae. aegypti* colony mosquitoes originally collected in Cairns. From these mosquitoes, the full segment sequences corresponding to the glycoprotein, nucleocapsid and RNA-dependent RNA polymerase of Phasi Charoen-like virus (PCLV) and Humaita-Tubiacanga virus (HTV), an unclassified +ssRNA virus were detected, consistent with detections in *Ae. aegypti* elsewhere in the world[29,59,60] (Table S3).

Table 3
Detection of other viral sequences in Australian mosquitoes.

Mosquito Species	Pool ID	Virus	E-value	Identity (%)	Query Cover (%)	Closest Match	Genbank Accession number
Cx. quinquefasciatus	HS042	CILV1	0.0	97.51	100	CILV1	PP066165
Cx. quinquefasciatus	HS003	CILV4	0.0	96.55	100	CILV4	PP076714
Ae. aegypti	Cairns WT male	PCLV	0.0	97.37	100	PCLV strain 19-7282-1, S segment	MN866293
Ae. aegypti	Cairns WT male	PCLV	0.0	96.3	100	PCLV strain 19-7282-1, M segment	MN866262
Ae. aegypti	Cairns WT male	PCLV	0.0	98.54	99	PCLV, Zhanjiang01 strain, L segment	MF614132
Ae. aegypti	Cairns WT male	HTV	0.0	97.15	100	HTV KS31 strain, RdRp	OQ305254
Ae. aegypti	Cairns WT male	HTV	0.0	98.14	100	HTV KS44 strain, capsid	OQ305244

Generation of CFAV and CxFV infectious clones through CPER

Both wild-type (WT) CxFV (isolate HS045) and CFAV (isolate Cairns WT male) replicated poorly in cell culture, with $TCID_{50}$ values typically ranging between $10^{3.97}$ $TCID_{50}$/ml to $10^{5.3}$ $TCID_{50}$/ml. Deep sequencing of the respective mosquito homogenates revealed co-infections with CFAV, HTV and PCLV in the *Ae. aegypti* samples, while Culex iflavivirus was detected in the *Cx. quinquefasciatus* samples (Table S1), suggesting the observed reduction in viral replication of CxFV HS045 and CFAV Cairns WT male was due to the presence of other competing ISVs. To obtain a clean and high-titre viral preparation, infectious clones based on the full-length sequences of isolates CxFV HS045 and CFAV Cairns WT male were generated using the CPER protocol (Fig. 3A). As initial attempts to launch the CFAV and CxFV infectious clones via CPER with the typically used minimal OpIE2 promotor (OpIE2-CA) were unsuccessful (Fig S1A), the *Ae. aegypti* polyubiquitin promoter (AePUb), previously shown to induce successful transcription in mosquito cells[61,62], was used. As the exact transcription initiation site of the *Ae aegypti* polyubiquitin promoter has not been elucidated, to ensure the authentic 5′ ends of both the CFAV and CxFV genomes, a hammerhead ribozyme sequence was included immediately after the polyubiquitin promoter[47]. The use of this promoter system resulted in the successful recovery of infectious clones of both CFAV and CxFV (Fig. 3B). In each case, identification of a silent point mutation in the NS4B region confirmed that the replicating virus was the infectious clone and not wild-type virus (Fig S1B). These data represent the first recovery of a CFAV infectious clone and the creation of a CxFV infectious clone by CPER. Both CFAV and CxFV infectious clones replicated

efficiently, reaching peak titres above10^8 $TCID_{50}$/ml and approximately $10^{8.3}$ $TCID_{50}$/ml 5 dpi when infected at an MOI of 0.1 (Fig. 3C) and displayed typical orthoflavivirus virion morphology when assessed under TEM (Fig. 3D). Both infectious clones induced CPE in C6/36 cells, consistent with previous reports in literature[12,63], with CFAV causing prominent syncytial CPE and CxFV inducing moderate destruction of the cell monolayer (Fig. 3E).

CFAV and CxFV are antigenically distinct from other orthoflaviviruses

Antigenic analyses of classical ISFs remains limited to date, predominantly due to lack of antibody tools. To assess the antigenic relationships between CFAV, CxFV and other orthoflaviviruses, and to develop crucial research tools to specifically detect CFAV proteins, mice were injected with CFAV proteins and a panel of hybridomas secreting mAbs reactive to CFAV produced and characterised (Table 4, Fig. 4A). Despite replicating efficiently in C6/36 cells, $CFAV_{ic}$ proved difficult to purify (data not shown). Therefore, CFAV prM and E immunogens were provided from a purified preparation of BinJ/CFAV-prME (bCFAV) chimeric virions. A total of five hybridomas secreting mAb reactive to CFAV were recovered, demonstrating clear cytoplasmic staining of $CFAV_{ic}$-infected cell monolayers when assessed by IFA (Fig. 4A, Fig. 4B, Table 4). Three of the mAbs were shown to be of the IgG1 isotype (CF3-2D5, CF3-5G4, CF3-1D6, CF3-5B9), while one was an IgM (CF3-3D1), and one an IgG3 (CF3-3E10) (Table 4).

By Western blot analysis, one mAb (CF3-2D5) bound prM (~ 25 Da), while three mAbs (CF3-1D6, CF3-5B9, CF3-5G4) bound the E protein (~ 50 kDa) (Fig S2). The binding specificity of the remaining two mAbs (CF3-3E10 and CF3-3D1) could not be determined, indicating that these mAbs could bind SDS-sensitive epitopes. None of the mAbs bound the CFAV proteins under reducing conditions, suggesting that each of the mAbs binds a confirmational epitope (Table 4).

Surprisingly, one anti-CFAV mAb, CF3-3D, also bound CxFV. Cross-reactivity assessment expansion to assess the binding of this mAb to other classical ISFs determined that it also effectively detected Palm Creek virus (PCV) and Paramatta River virus (PaRV) (Fig. 4C). Given the ability of mAb CF3-3D1 to bind four different classical ISF species with amino acid similarity within the prM and E proteins ranging from 45.0% to 73.4% (Table S7), we hypothesised that this mAb may have contact residues within the E protein fusion loop. Indeed, CFAV, CxFV, PCV and PaRV have a conserved “FKW” motif at residues 106–108 of the E protein. To provide further strength to this hypothesis, we assessed the prM and E proteins of three additional classical ISFs by expressing the prM and E genes of NIEV, KRV and NAKV by constructing chimeric viruses using the BinJV backbone. We have shown extensively these chimeras authentically mimic the structure and antigenicity of wild-type particles[6,64] and thus, these chimeras enabled us to extend comparative analysis across ISFs. Successful recovery of each chimera was confirmed by reactivity with pan-orthoflavivirus NS1-specific mAb 4G4 and sequencing across the capsid-E-NS1 junctions. Of these three additional viruses, NIEV retains the FKW motif, but a K107E substitution exists for KRV and two substitutions within this region occur for NAKV such that the motif is LEW (Table S8). mAb CF3-3D1 bound NIEV, but not to KRV, nor to NAKV (Fig. 4B), suggesting that this hypothesis is plausible, however, definitive evidence for binding of this mAb to this region would require mutagenesis of the FKW residues. As there were variable dissociation constants (Kd = 2.956–8.591) for mAb CF3-3D1 for the classical ISFs, it is likely that other contact residues for this mAb exist outside of the core “FKW” motif (Fig S7) and this is similarly consistent with the inability of the mAb to bind to reduced antigen.

Table 4
Characterisation of anti-CFAV mAbs.

mAb	DTT-sensitivity	Viral Target[A]	Isotype	mAb cross-reactivity in ELISA[B]										
				classical ISF							dual host-affiliated ISF			VIF
CFAV				CxFV	PCV	PaRV	NIEV[C]	KRV[C]	NAKV[C]	BinJV	NHUV[C]	ILOV[C]	WNV_{KUN}	
CF3-2D5	Yes	prM	IgG_1	+	-	-	-	-	-	-	-	-	-	-
CF3-3D1	Yes	NR	IgM	+	+	+	+	+	-	-	-	-	-	-
CF3-3E10	Yes	NR	IgG3	+	-	-	-	-	-	-	-	-	-	-
CF3-5B9	Yes	E	IgG_1	+	-	+	-	+	NT[D]	NT[D]	-	-	-	-
CF3-5G4	Yes	E	IgG_1	+	-	+	-	-	NT[D]	NT[D]	-	-	-	-
Assay controls														
4G4[48]	NA	NS1	IgG_1	-	-	-	-	+	+	+	+	+	+	+
3G1*[65]	NA	dsRNA	IgM	+	+	+	+	-	-	-	-	-	-	+
2G4*[65]	NA	dsRNA	IgM	+	+	+	+	-	-	-	-	-	-	+

[A] Determined by Western blot analysis. E = 50 kDa protein; prM = 25 kDa protein; NR = no reaction

[B] + = reactive; - = non-reactive. A result was considered positive if it exceeded twice the optical density (OD) of the same mAb dilution on mock infected cells.

[C]NIEV, KRV, NAKV, NHUV and ILOV consisted of chimeric virus containing the BinJV backbone and the prM and E genes from the selected ISFs.

[D] NT = Not tested

**Aedes*-associated dual-host affiliated ISFs, including BinJV-based chimeras, are negative with acetone fixation[40,65]

To further extend antigenic analyses, $CFAV_{ic}$ and $CxFV_{ic}$-infected cell monolayers were probed with a suite of mAbs generated to other Australian classical ISFs (PaRV[66], PCV[67]) or a dual host-affiliated ISF (BinJV[6,10]). In each instance, the mAbs failed to detect CFAV and CxFV, except for anti-dsRNA mAbs 3G1 and 2G4 (Table 5). These data indicated that CFAV and CxFV are antigenically distinct from other Australian ISFs, consistent with our previous analysis of classical ISFs that showed negligible antigenic similarity between these viruses[10].

Table 5: Binding assessment of anti-ISF mAbs to CFAV and CxFV by other mAbs derived to Australian ISFs by ELISA.

		mAb reactivity in ELISA*				
mAb	**Viral Target**	**CFAV**	**CxFV**	**PCV**	**PaRV**	**BinJV**
3G1[#39]	dsRNA	+	+	+	+	-
2G4[#39]	dsRNA	+	+	+	+	-
BJ-6E6[6]	pan-Flavi E	-	-	-	-	+
BJ-2B11[6]	BinJV E	-	-	-	-	+
BJ-7G10[6]	BinJV NS1	-	-	-	-	+
5G12[30]	PCV E	-	-	+	-	-
5D8.1[30]	PaRV E	-	-	-	+	-
2G3.1[30]	PaRV prM	-	-	-	+	-
5B7[30]	PaRV prM	-	-	-	+	-
7D11[30]	PaRV E	-	-	-	+	-
3G7[30]	PaRV E	-	-	-	+	-

* Cut-off for a positive result was 2x OD of the same dilution of mAb on mock cells. "+" = positive result and "-" = negative result

[#]*Aedes*-associated dual-host affiliated ISFs, including BinJV-based chimeras, are negative with acetone fixation[40,65]

Detection of infection and dissemination of CFAV within Ae. aegypti mosquitoes

Virulence of the CFAV infectious clone *in vivo* was demonstrated by dissemination of the virus through mosquitoes following IT microinjection (Fig. 5). The anti-dsRNA mAb 2G4 was used to probe CFAV- and PBS-injected *Ae. aegypti* mosquitoes at 8 days post-inoculation. This assay revealed widespread infection through the hemolymph (Fig. 5A), with intense staining in neural tissues of the head (Fig. 5D), consistent with localised viral replication. Infection in the midgut and ovaries indicates the potential for superinfection exclusion (SIE) and vertical transmission (VT), although the signal was not uniformly distributed across tissues. No specific staining was seen when CFAV-infected mosquitoes were probed with anti-CFAV mAbs CF3-2D5 or CF3-3D1, indicating that this mAb is unsuitable for IHC (Fig S3).

Discussion

The isolation and characterisation of ISFs provides important insight into the evolutionary history of the *Orthoflavivirus* genus and provide novel tools for biocontrol and the development of vaccine and diagnostic antigens[2,6]. This study reports the first isolation of CFAV and CxFV from Australian mosquitoes and establishes new molecular and immunological tools for their investigation, including the first infectious clone of CFAV, a CPER-derived infectious clone of CxFV, and the first CFAV and broader ISF-reactive mAbs. These resources substantially expand the experimental toolkit for lineage I ISFs and provide new opportunities to investigate their biology, host-restriction, virus-mosquito interactions and biotechnological applications. As the prototypic *Aedes*- and *Culex*-associated classical ISFs, CFAV and CxFV have been widely used to study superinfection exclusion and vertical transmission, making these resources broadly valuable to the field.

While CFAV and CxFV have been shown to infect different mosquito species they are generally restricted to the mosquito genus of their first isolation i.e. *Aedes* and *Culex* respectively[2,17–24,29,68–71]. CFAV has been predominantly detected in *Ae. aegypti* mosquito populations worldwide, but also detected or isolated from other *Aedes* species, including *Ae. albopictus*[72], *Ae. caballus*[73] and occasionally in other genera, including *Cx. species*[72]. Consistent with detections in wild-caught mosquitoes, CFAV has also been identified in numerous laboratory *Ae. aegypti* colonies[14,74], where persistent infections are thought to be maintained by highly efficient vertical transmission. Thus, the detection of CFAV in the colony-reared *Ae. aegypti* Cairns-derived mosquitoes were not unexpected, with recent metagenomics studies detecting CFAV RNA in wild-caught *Ae. aegypti* that were captured in a similar region to the origin of the colony[29].

To the best of our knowledge, the detection and isolation of CxFV from several pools of *Cx. quinquefasciatus* mosquitoes from Brisbane is the first report of this virus within Australia and is consistent with the widespread distribution of this virus in *Culex* mosquitoes worldwide. Since its initial description, CxFV has been reported from *Cx. quinquefasciatus* populations across Asia, Africa, the Americas and the Caribbean, where it is frequently detected and maintained through efficient vertical transmission[14,75,76]. It has also been frequently detected in several other medically important *Culex* species, including *Cx. pipiens*[69], *Cx. tritaeniorhynchus*[69], *Cx. tarsalis*[63] and *Cx. restuans*[75], suggesting that the virus is broadly adapted to mosquitoes within this genus. The high sequence similarity of the Australian isolate to CxFV strains circulating throughout Asia further supports the close evolutionary

relationship between these viruses and is consistent with the geographic clustering described for CxFV lineages[63,77,78]. Combined, these findings indicate that CxFV is a well-established component of the *Culex* mosquito virome across the Asia-Pacific region and reinforces the need to determine whether persistent CxFV infection influences the vector competence of Australian *Cx. quinquefasciatus* for medically important orthoflaviviruses, including JEV and WNV_{KUN}.

The detection of ISVs in colony-reared mosquitoes in this study is also noteworthy, as this can alter the outcomes of studies on vector competence or virus-mosquito interactions[36]. Previous studies have shown the persistent infection of mosquito populations with some ISFs can alter the transmission of orthoflavivirus pathogens by the vector[4,14]. Indeed, persistent CxFV infection in *Cx. pipiens* mosquitoes provides early suppression of WNV replication[53], while in a different study, CxFV was reported have no effect on or significantly enhanced the transmission of WNV in *Cx. quinquefasciatus* mosquitoes, dependent on the origin of the mosquito colony[79]. Similarly, the Aag2 cell line-derived CFAV strain has been reported to promote DENV-2 infection in *Aedes* mosquito cell lines[80], while a mosquito isolate from Thai mosquitoes reduced replication of DENV-1 *in vitro* and negatively affected the dissemination of both DENV-1 and ZIKV *in vivo*[74]. Considering these conflicting results, elucidation of the mechanisms involved in ISF interactions with pathogenic orthoflaviviruses at the cellular level and in the whole mosquito may provide additional, novel, biological control mechanisms of pathogenic orthoflaviviruses. Interestingly, other ISVs have similarly been attributed to affecting pathogen transmission. Recent studies have also demonstrated that the presence of HTV or the bunyavirus, PCLV, increased transmission of DENV and ZIKV to a vertebrate host via *Ae. aegypti* mosquitoes[81]. Consistent with the frequent detection of HTV and PCLV by *Ae. aegypti*, sequencing the RNA from a pool of male mosquitoes collected from Cairns revealed the presence of RNA from both PCLV and HTV.

A key outcome of this study is the development of the first reported infectious clone of CFAV and the successful recovery of a CxFV infectious clone using CPER. While the modified OpIE2-CA (*Orgyia pseudotsugata* immediate early release 2) insect promoter has been routinely used in our laboratory to launch infectious clones of diverse mosquito-borne viruses, including orthoflaviviruses, alphaviruses and mesoniviruses[46,82,83], repeated attempts to recover CFAV using this promoter OpIE2-CA promoter were unsuccessful, despite its proven utility. Replacement of the OpIE2-CA promoter with the *Ae. aegypti* polyubiquitin promoter, which has been shown to drive robust transcription in mosquito cells[47,61], resulted in successful recovery of infectious CFAV using the same backbone PCR fragments that were used in attempts with the OpIE2-CA promoter. These data highlight promoter selection as a critical consideration in the design of mosquito cell-based reverse genetics systems. Notably, the same polyubiquitin promoter also enabled successful recovery of a CxFV infectious clone, demonstrating its broader applicability for classical ISFs.

Reverse genetics systems have previously been developed for several lineage I ISFs, including CxFV[84], PaRV[11] and NIEV[33], and consistently recapitulate the biological properties of their wildtype parental counterpart. An infectious clone of CxFV constructed using the traditional plasmid-based approach produced recombinant virus with growth kinetics, cell cytopathic effects and virion morphology indistinguishable from the parental isolate[84], although viral replication was assessed primarily by qRT-PCR owing to the absence of suitable antibody reagents. Similarly, infectious clones of PaRV and NIEV exhibited replication kinetics comparable to their respective wild-type parental viruses[11,33], further demonstrating the fidelity of reverse genetics systems for classical ISFs. Consistent with these observations, the infectious clones for CFAV and CxFV established efficient infection in mosquito cells and induced characteristic CPE reported during infection of the wild-type viruses[12,69,79,80]. The reverse genetics systems established here therefore provides a robust platform for investigating the molecular determinants of superinfection exclusion, tissue tropism and vertical transmission, while enabling precise genetic manipulation without the confounding influence of co-infecting viruses that may be present in field- or colony-derived isolates. Although reverse genetics systems for CxFV have previously been described, the successful recovery of CxFV using CPER further demonstrate the versatility and robustness of this approach for future *in vitro* and *in vivo* studies of ISFs.

Prior to this study, comprehensive antigenic analyses of classical ISFs were limited[85], largely reflecting the paucity of virus-specific mAbs to classical ISFs available as research tools. Although polyclonal sera has been developed against some ISFs[79,85], their utility across classical ISFs appears limited. For example, hyperimmune sera raised against an arbovirus cocktail detected several dual host-affiliated ISFs, but showed little or no reactivity with classical ISFs[85] Our team has successfully developed mAbs for other ISFs, including for PCV[67], PaRV[11], Karumba virus (KRBV)[42] and BinJV[6,10], however, none of these mAbs recognised CFAV, nor CxFV. As virus-specific antibodies are particularly important for detecting, quantifying and localising ISF infection in cell culture and mosquito tissues, we generated the first panel of mAbs to CFAV, addressing a significant gap in the field.

Of the panel of anti-CFAV mAbs generated, two bound CFAV exclusively, while an additional two also showed some reactivity to PCV and in one case, NIEV. Unexpectedly, one mAb, CF3-3D1, recognised five classical ISFs, including CxFV, suggesting epitope conservation between these two ISF species. As we did not have access to isolates of several lineage I ISFs that are exotic to Australia, including NIEV, KRV and NAKV, we used CPER to construct chimeric viruses by splicing the prM and E gene sequences of these viruses into the BinJV genome. We have previously demonstrated by high-resolution cryo-EM, these BinJV chimeric virus particles authentically reproduce the antigenic structure of the wild-type virion[64], providing a robust system for comparative antigenic analysis. To our knowledge, the broad recognition of mAb CF3-3D1 represents the first detailed demonstration of antigenic conservation across multiple classical ISF species and contrast with our previously generated anti-ISF E, prM and NS1-reactive mAbs which showed little or no cross-reactivity between ISFs.

The broad reactivity of mAb CF3-3D1 may reflect recognition of a conserved region within the E protein fusion loop. Vertebrate-infecting orthoflaviviruses and dual host-affiliated ISFs are frequently recognised by group-reactive mAbs such as 4G2 and BJ-6E6, which target a highly conserved epitope within the E protein fusion loop; however, these antibodies do not recognise classical ISFs[10]. This difference is consistent with characteristic sequence variation within the classical ISF fusion loop. In many vertebrate-infecting orthoflaviviruses and dual host-affiliated residues 106–108 comprise the

highly conserved G106, L107, F108 motif[86,87], whereas many classical I ISFs examined here contain F106, K/E107 and W108. Notably, the mAb CF3-3D1-reactive viruses, CFAV, CxFV, NIEV, PCV and PaRV share complete amino acid sequences across E residues 98–109 of the E protein (Table S7). In contrast, KRV differs at residue 107 (K107E) and was not recognised by mAb CF3-3D1. NAKV, which contains the same K107E substitution, together with F106L, was also not recognised. This strong association between sequence conservation and antibody binding suggests that residues within, or structurally associated with this region may contribute to the CF3-3D1 epitope, with residue 107 representing a particularly strong candidate determinant. This hypothesis will require confirmation through targeted mutagenesis and epitope mapping studies.

The sequence conservation observed here also predicts that mAb CF3-3D1 may recognise additional classical ISFs that share the CFAV sequence across E residues 98–109, including *Culex theileri* flavivirus, Quang Binh virus and Hanko virus, but this would require empirical validation. Collectively, these findings demonstrate that broadly cross-reactive mAbs can be generated against classical ISFs and reveal previously unrecognised antigenic conservation among viruses within this clade. mAb CF3-3D1 therefore provides both a potentially valuable research reagent for detecting diverse classical ISFs and a tool for defining conserved antigenic features of their E proteins.

To further investigate these observations in the context of CFAV and using what is known to be a pure CFAV stock due to the use of our infectious clone, we IT-injected *Ae. aegypti* mosquitoes and performed IFA to localise the virus. Here, dsRNA staining using mAb 2G4[39,65] revealed discrete cytoplasmic foci consistent with active viral replication following IT injection. Replication-associated dsRNA was detected in multiple tissues using mAb 2G4[40,65], including the midgut epithelium and ovarian tissue, both of which represent biologically important sites for arbovirus establishment and vertical transmission, respectively. These results are consistent with previous reports demonstrating dissemination of the Galveston strain of CFAV to the midgut and ovaries of Mexican *Ae. aegypti* mosquitoes following IT injection, as detected by fluorescent in situ hybridisation[88]. The presence of dsRNA within these tissues supports successful dissemination of our CFAV infectious clone beyond the injection site and indicates a consistent/persistence infection and the reported transovarial maintenance in naturally infected *Ae. aegypti* populations[89]. Despite extensive optimisation, the CFAV antibodies generated here failed to lead to staining in infected tissues. The discrepancy between the CFAV-specific mAbs and dsRNA staining indicates that infection was successfully established, but that the epitopes recognised by the anti-CFAV mAbs were likely altered or masked during formaldehyde fixation, preventing reliable antigen detection.

While interactions between arboviruses and ISFs have been investigated *in vitro* and *in vivo* in SIE experiments, comparatively little is known about the dissemination dynamics of ISFs within the mosquito host. Koh et al. demonstrated qRT-PCR that IT injection with $1x10^4$ $TCID_{50}$/ml of PCV resulted in widespread dissemination, with viral titres reaching up to 1×10^9 Log_{10} genome copies/tissue in the carcass, midgut, and ovaries of both *Ae. aegypti* and *Ae. albopictus* by 4 dpi and peaking at 10 dpi and 14 dpi respectively [90]. Similarly, CxFV has been shown by IFA using murine-derive polyclonal sera (pAb) to disseminate to the midgut and head tissues when IT injected[79]. However, it remains unclear if the virus stocks used to generate the pAb were free of co-infecting viruses, which may influence the interpretation of these findings. In contrast, we previously observed that when PCV at 10^5 $TCID_{50}$/ml was injected intrathoracically into *Cx. annulirostris* mosquitoes, the mean virus titre in the mosquito bodies reached 10^5 $TCID_{50}$/ml, but the virus was detected by IHC using virus-specific pAb, only within epithelial cells lining the midgut at 7 dpi[4]. Likewise, we have previously similarly shown localisation of PaRV and KRBV exclusively to the midgut tissue by IHC in naturally infected mosquitoes[42,91]. The differing dissemination patterns observed across studies may reflect a combination of factors, including differences in detection sensitivity, virus species, mosquito host and infection route. In particular, comparisons between experimentally inoculated and naturally infected mosquitoes should be interpreted cautiously, as the observed tissue distribution may be influenced by both the infection context and the sensitivity of the detection method employed.

In addition to the isolation of CxFV, the complete ORFs of two iflaviviruses were also identified in *Cx. quinquifasciatus* collected from Brisbane. Iflaviviruses are non-enveloped, positive strand viruses that are related to picornaviruses, and often infect arthropods[92]. Previous metagenomic studies on RNA extracted from mosquitoes captured in the Kimberly region of north-western Australia have identified novel iflavivirus genomes in *Cx. annulirostris*[58]. However, phylogenetic analyses showed that the two Brisbane iflavivirus sequences differed significantly from the Kimberly sequences and were more closely related to Culex iflavivirus-like 1 and Culex iflavivirus -like 4 sequences previously detected in *Culex* species captured in California, USA[57]. They likely represent the first isolation of viable strains of these viruses. The apparent isolation of two iflaviruses from *Cx. quinquifasciatus* from Brisbane also provides an opportunity for further studies to characterise these viruses and determine their host specificity (*in vitro* and *in vivo*) and the mechanism of transmission as well as their interaction with pathogenic arboviruses in co-infected mosquito vectors.

In summary, we have obtained the first Australian isolates of CxFV and CFAV, generated infectious clones via CPER for these viruses and produced a suite of mAbs reactive to CFAV and CxFV, with one also detecting other lineage I ISFs. These innovations mark a significant advancement in the production of tools to promote our understanding of mosquito-virus interactions, which are crucial for elucidating the dynamics of arbovirus transmission. Prior studies have demonstrated the potential of ISFs to limit arbovirus dissemination in mosquitoes and mosquito cells[35,74,93]; however, the *in vivo* infection dynamics of mosquito-virus interactions remain largely unexplored [35,93,94]. By providing novel tools for *in vitro* and *in vivo* investigations, our findings lay the foundation for advancing the use of CFAV and CxFV as biocontrol agents and improving our understanding of their role in mosquito systems.

Declarations

Competing Interests

Data included in this publication are based on a patent (WO/2018/176075), on which, N.D.N, R.A.H, J.H-P and J.J.H are inventors.

Author Contribution

Experimental design: A.J.P, R.A.H, L.E.H, J.H-P, J.J.HExperiments: A.J.P, I.E.M, L.D.H, R.A.J, M.S.A, D.P, C.C.W.A, J.J.HProvision of reagents: J.R.P, R.H.P, N.D.N, D.J.R, J.DFunding: R.A.H, L.E.H, J.H-P, J.J.HWriting, original draft preparation: A.J.P, R.A.H, L.E.H, J.H-P, J.J.HWriting, review and editing: all authors

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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