A New Insect-Specific Flavivirus from Northern Australia Suppresses Replication of West Nile Virus and Murray Valley Encephalitis Virus in Co-infected Mosquito Cells

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Abstract

Recent reports of a novel group of flaviviruses that replicate only in mosquitoes and appear to spread through insect populations via vertical transmission have emerged from around the globe. To date, there is no information on the presence or prevalence of these insect-specific flaviviruses (ISFs) in Australian mosquito species. To assess whether such viruses occur locally, we used reverse transcription-polymerase chain reaction (RT-PCR) and flavivirus universal primers that are specific to the NS5 gene to detect these viruses in mosquito pools collected from the Northern Territory. Of 94 pools of mosquitoes, 13 were RT-PCR positive, and of these, 6 flavivirus isolates were obtained by inoculation of mosquito cell culture. Sequence analysis of the NS5 gene revealed that these isolates are genetically and phylogenetically similar to ISFs reported from other parts of the world. The entire coding region of one isolate (designated 56) was sequenced and shown to have approximately 63.7% nucleotide identity and 66.6% amino acid identity with its closest known relative (Nakiwogo virus) indicating that the prototype Australian ISF represents a new species. All isolates were obtained from Coquillettidia xanthogaster mosquitoes. The new virus is tentatively named Palm Creek virus (PCV) after its place of isolation. We also demonstrated that prior infection of cultured mosquito cells with PCV suppressed subsequent replication of the medically significant West Nile and Murray Valley encephalitis viruses by 10–43 fold (1 to 1.63 log) at 48 hr post-infection, suggesting that superinfection exclusion can occur between ISFs and vertebrate-infecting flaviviruses despite their high level of genetic diversity. We also generated several monoclonal antibodies (mAbs) that are specific to the NS1 protein of PCV, and these represent the first ISF-specific mAbs reported to date.

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Introduction

Flaviviruses are responsible for a number of important mosquito-borne diseases of humans and animals in Australia, including dengue, Murray Valley encephalitis and Japanese encephalitis (JE) [1]. Dengue, JE, yellow fever and West Nile fever are also major medical problems around the world [2]. Flaviviruses are a group of small, enveloped viruses that contain a positive-sense RNA genome with a single open reading frame (ORF) which is flanked by 5’ and 3’ untranslated regions (UTRs). The ORF is translated as a single polyprotein, which is cleaved by viral and cellular proteases into three structural (C, prM and E) and seven non-structural proteins (NS1-NS5).

Flaviviruses are usually transmitted between arthropods and vertebrates and rely on replication in both of these hosts for their natural transmission cycle. In 1975, Stollar and Thomas reported the isolation of an unusual virus (cell fusing agent virus; CFAV) from mosquito cell cultures [3]. Further analysis revealed that CFAV is a distant relative of members of the flavivirus genus, but did not replicate in vertebrate cells. CFAV and similar viruses - Kamiti River virus (KRV) and Culex flavivirus (CxFV) - were subsequently isolated from mosquitoes in the wild and shown to belong to a distinct “insect-specific” flavivirus (ISF) lineage [4–6]. With the advent of improved molecular tools for viral detection, several new species of ISF including Aedes flavivirus (AeFV) [7,8], Quang Binh virus (QBV) [9], Nakiwogo virus (NAKV) [10], Chaoyang virus (Genbank accession number FJ803471 – Wang et al., 2009), Lammi virus [11], Nounane virus [12], Calbertado virus [13] and Culex theleri flavivirus (CITFV [14]), have since been isolated from various regions of the world.

Data from several studies indicates that at least some ISFs are maintained in nature in the absence of a vertebrate host by vertical
transmission from female mosquitoes to their progeny [15–17]. A lack of a direct association of these viruses with disease has largely seen ISFs ignored to date, however, recent reports by Kent et al. (2010) [16] and Bolling et al. (2012) [17] suggesting that co-infection with CxFV may enhance or suppress transmission of West Nile virus (WNV) in some vectors has created intense interest in the interaction of ISFs with other flaviviruses in mosquito cells. In this paper, we report the isolation and phylogenetic analysis of a new ISF detected in mosquito pools from northern Australia and the generation of ISF-specific recombinant proteins and monoclonal antibodies. We also provide in vitro evidence of “super-infection exclusion” of heterologous flaviviruses in cell cultures previously infected with this new virus.

Materials and Methods

Ethics Statement

The mouse work in this study was carried out under conditions approved by The University of Queensland Animal Ethics Committee (Animal Ethics Number 299/10). Surgery was performed under ketamine/Xylazine and all efforts were made to minimize suffering.

No specific permits were required for the described field studies and no specific permissions were required for the locations/activities for mosquito trapping because they are public lands and are not privately owned or protected in any way. The sites of mosquito trapping are those where the Northern Territory Department of Health conducts regular mosquito monitoring and has done so for many years. These field studies did not involve endangered or protected species.

Trapping and Processing of Mosquitoes

Mosquitoes were trapped in March to June, 2010 from various sites around the Northern Territory of Australia. These sites included Darwin, Katherine, Alice Springs, Alyangula, Groote Eylandt, Jabiru and the McArthur River Mine. The mosquitoes were trapped by E.V.S. dry ice baited light traps [19], sorted to species in pools of 1 to 50 as previously described [20] and stored at −80°C until processed. All of the mosquitoes were female. Mosquito pools were homogenized for RNA extraction and virus isolation as previously described [21].

Viral RNA Detection and Isolation from Mosquito Homogenates

Mosquito homogenates were screened for the presence of flavivirus RNA using the pan-flavivirus specific primers FU2 and cFD3 [22] using the methods described in Blitvich et al. (2009) [23]. Briefly, viral RNA was extracted from 200 μl of mosquito homogenate using the Qiagen RNAeasy extraction kit as per the manufacturer’s instructions and purified RNA was eluted in 50 μl of nuclease-free water. Five μl of purified RNA was then tested by RT-PCR (SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase, Invitrogen) for the presence of flavivirus RNA. A second aliquot of mosquito homogenate from PCR-positive pools was inoculated onto monolayers of C6/36 cells and incubated at 28°C for 5–7 days. Two hundred μl of culture supernatant was collected from the inoculated cultures and then total RNA was extracted and tested by RT-PCR as described above. Aliquots of culture supernatant from PCR-positive cultures were then stored at −80°C for further analysis.

Sequencing of Viral Isolates

Initial sequencing of part of the NS5 genes for each viral isolate was performed on the RT-PCR product generated from the primer pair FU2 and cFD3 [22] using the protocol described above. The nucleotide sequence of the entire coding region of one PCV isolate (designated isolated 56) was determined by a combination of gene walking using a series of primers designed from PCV-derived nucleotide sequence and from regions of conserved sequence of published ISF genomes (NAKV, QBV, CxFV) and either a two-step RT-PCR using Superscript III reverse transcriptase (Invitrogen) and Phusion high fidelity DNA polymerase (Finnzymes) or the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen). The 5′ end of the sequence was amplified using the GeneRacer kit (Invitrogen) according to the manufacturer’s instructions. The amplifications were purified by agarose gel electrophoresis and extracted using the NucleoSpin® Gel and PCR Clean-up kit (Macherey Nagel). The purified DNA fragments were sequenced at the Australian Genome Research Facility (Brisbane, Queensland). For some regions of the genome, a cloning passage was performed and sequencing performed directly on purified plasmid.

Sequence Alignments and Phylogenetics

Virus sequences were aligned using the AlignX component of the Vector NTI suite (Invitrogen), and then inspected and edited using BioEdit [24]. Nucleotide and amino acid identities were calculated with BioEdit. Phylogenetic trees were constructed using the Phylogroup of programs (neighbour joining trees) [25] and PhyML (maximum likelihood trees) [26]. Bootstrapped maximum likelihood trees were constructed using the NS3/3’UTR and complete open reading frames (ORF) of a selection of ISFs. The trees were mid-point rooted and are based on 1000 replicates. Specific parameters are available from the authors on request.

Cell and Virus Culture

C6/36 cells (ATCC CRL-1660) were cultured in RPMI 1640 with 5–10% fetal bovine serum (FBS) and incubated at 28°C. The mammalian cells, African Green Monkey Kidney (Vero; ATCC CCL-81), baby hamster kidney (BHK-21; ATCC CCL-10), porcine stable equine kidney (PS-EK; [27]) and human adenocarcinoma (SW-13 CCL-105) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2% FBS, while the hybridoma cell lines produced in this study were grown in hybridoma serum free medium (Invitrogen) initially supplemented with 20% FBS and then weaned to serum-free culture for antibody production. COS-7L cells (Invitrogen) were maintained in RPMI 1640 with 2% FBS. All mammalian cells were incubated at 37°C with 5% CO₂. All media were supplemented with 50 U penicillin/mL, 50 μg streptomycin/mL and 2 mM L-glutamine.

Each virus isolate was propagated by inoculating onto monolayers of C6/36 cells and incubation at 28°C for 5–7 days before harvest. The viral titre was determined by 50% tissue culture infective dose (TCID₅₀) assays using methods described by May et al. (2006) [28].

To enhance the visualisation of cytopathic effect (CPE), the growth medium on PCV-infected C6/36 monolayers was replaced with medium that had been adjusted to pH 6.0 using sterile-filtered 1M MES (2-(N-morpholino)ethanesulfonic acid hydrate) buffer. Images of the monolayers were captured 24 hr after changing the medium.
Analysis of Virus Replication in Mammalian Cell Lines

Stocks of PCV (isolate 56) and WNV (Kunjin virus MAR16; WN89KUNV), prepared in C6/36 cells, were used to inoculate a panel of mammalian cell lines commonly used in the culture of flaviviruses. These included Vero, BHK-21, PS-EK and SW-13 cells. After culturing for 7 days, the inoculated cells were examined for the presence of CPE and cell monolayers were tested for viral RNA by RT-PCR using the FV2/cFD3 primer pair.

Morphology of Viral Particles under Transmission Electron Microscopy (TEM)

PCV particles from C6/36 cell culture supernatant collected at 5 days post-inoculation were concentrated through a high molecular weight cut-off (300 K) centrifugal concentrator (Sartorius). Concentrated samples were placed onto a formvar-coated copper grid, negatively stained with 1% uranyl acetate and viewed in a JEOL1010 transmission electron microscope.

Production and Characterisation of Monoclonal Antibodies to Palm Creek Virus

Hybridomas were generated to PCV proteins by immunizing an adult female BALB/c mouse with partially purified viral antigen. Briefly, PCV particles from PCV-infected C6/36 cell culture supernatants were concentrated through a high molecular weight cut-off (300 K) filter or by ultracentrifugation at 28,000 rpm (SW41Ti rotor Beckman) for 4 hr at 4°C. Aliquots of these preparations, along with Titre-Max Gold antiserum (Sigma-Aldrich) diluted according to the manufacturer’s instructions were used to immunize the mouse subcutaneously with three doses given at 14–28 day intervals. The mouse was boosted with a fresh preparation of concentrated PCV viral antigen (no adjuvant) about five months later and the spleen harvested three days following this fusion. The spleen of the spleen cells with myeloma cells was performed as previously described [29]. Hybridomas secreting antibodies reactive to PCV-infected C6/36 cells were identified by enzyme-linked immunosorbent assay (ELISA) using previously described methods [30]. However, in this case, incubation steps were performed at 37°C to enhance sensitivity of the assay. The hybridomas were cloned by limit dilution and harvested culture supernatants were concentrated through a high molecular weight cut-off (300 K) filter or by ultracentrifugation at 28,000 rpm (SW41Ti rotor Beckman) for 4 hr or 5 min at room temperature respectively.

Western blot and fixed-cell ELISA. The reactivity of the anti-PCV mAbs, were further tested for reactivity in immunofluorescent antibody assay (IFA), Western blot and fixed-cell ELISA. The reactivity of the anti-PCV mAbs to PCV viral lysates in Western blot was performed using previously published methods [30]. Each mAb was assessed for viral specificity by testing against a panel of vertebrate-infecting flaviviruses. These included Vero, BHK-21, PS-EK and SW-13 cells. After culturing for 7 days, the inoculated cells were examined for the presence of CPE and cell monolayers were tested for viral RNA by RT-PCR using the FV2/cFD3 primer pair.

Immunofluorescence Assay (IFA)

C6/36 cells were grown on glass coverslips and infected with PCV or WNVKUNV at a multiplicity of infection (M.O.I.) of 0.1, or sham-infected for 5 days, fixed in acetone and then blocked with 0.2% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 1 hr. Following incubation with the relevant mAbs in hybridoma cell culture fluid, the coverslips were washed with PBS and then stained with Alexafluor 488-conjugated goat anti-mouse IgG (H+L) (Invitrogen) and Hoechst 33342 nuclear stain (Invitrogen) for 1 hr or 5 min at room temperature respectively. Following another 3 washes in PBS, the coverslips were mounted onto glass microscope slides using ProLong Gold Anti-fade (Invitrogen) and viewed under the ZEISS LSM 510 META confocal microscope.

Expression of Recombinant PCV prM/E, NS1 and NS5 Proteins in Mammalian Cells

The genes for the PCV pre-membrane (prM) and envelope (E) (minus the predicted sequence for the transmembrane domain), NS1 and NS5 (lacking the 5’ coding sequences for the first 85 amino acids of the predicted protein for NS5) proteins were amplified by high fidelity RT-PCR and cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen) to generate three separate constructs – one each for prM/E, NS1 and NS5. For each, the 3’ end of the PCV gene was fused to the V5 epitope sequence followed by a polyhistidine sequence. The Japanese encephalitis virus (JEV) signal sequence from pcBWN [32,33] and the Kozak sequence [34] were inserted upstream of the prM and NS1 genes to ensure secreted expression of each of these proteins. Sequencing of the expression plasmid confirmed that the recombinant gene fragment was authentic and in-frame for correct translation.

PNGase F Digest and Analysis of Secreted Proteins

COS-7L cells were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. As a control for this experiment, recombinant WNV NS1 was produced following transfection of cells with a similar construct to pcBWN [32,33], except the prM/E genes were replaced with the gene for WNV NS1 (pcBWN-NS1). Cell lysates were harvested at 48 hr post-transfection by adding 150 μl per well (6-well plate) of BS9 lysis buffer (120 mM NaCl, 50 mM H3BO3, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, pH 9.0) to the cell monolayer. The cells were incubated with lysis buffer on ice for 15 min and then clarified by centrifugation at 12 000 g for 10 min at 4°C and stored at −20°C. For PNGase F digestion, 2 μl of 10% SDS was added to 15 μl of cell lysate and incubated at 95°C for 5 min. After heating, the samples were incubated at 4°C for 2 min before the addition of 1 μl 20% Octyl β-D-glucopyranoside, 2 μl G7 buffer (NEB), and 1 μl PNGase F (NEB). PNGase F digestion was carried out at 37°C for 2 hr, and the samples were analysed by Western blot to determine protein glycosylation indicated by the change in protein mobility as previously described [35].

To assess for secretion of the recombinant NS1 protein, culture supernatant was harvested 48 hr post-transfection and analysed by Western blot. For this Western blot, as well as the PNGase F Western blot, the recombinant PCV proteins were detected using anti-V5 mAb (Invitrogen), while the WNV NS1 protein was detected with the anti-NS1 mAb 4G4 [30].

Superinfection Exclusion Experiments

Monolayers of C6/36 cells were inoculated at 80% confluency with PCV (M.O.I. ≥1), or sham inoculated with media only, and allowed to incubate for 4–5 days. The cells were seeded into 24
well plates, some containing glass coverslips and incubated for a further 48 hr until cells had almost reached confluency. The growth medium was then removed and cells inoculated with the medically significant flaviviruses Murray Valley encephalitis virus (MVEV) and WNV (Kunjin virus MRMJh subtype – WNVKUNV) or the alphavirus Ross River virus (RRV) at an M.O.I. of 0.1 and incubated for 24 or 48 hours. At each time point, the titre of each secondary infecting virus in the culture supernatant was measured by 50% tissue culture infectious dose (TCID50) on PS-EK cells and the infectious titre compared with that in sham-infected controls. All samples were tested in triplicate. At each time-point, cell monolayers on coverslips or in wells were fixed in cold acetone. The replication of MVEV, WNVKUNV or RRV was examined by IFA staining using the flavivirus pan-reactive anti-NS1 mAb 4G4 [30] or RRV-specific mAbs G8 [36] and imaging performed using the IN CELL Analyzer (GE Healthcare Life Sciences). Co-staining of coverslips was performed by incubation with anti-PCV mAb 3D6 and Alexa-Fluor 488-labelled mAbs 4G4 or G8 (labelled using the Zenon Tricolour Labelling kit (Invitrogen) according to manufacturer’s instructions). In this instance, mAb 3D6 was detected with Alexa Fluor 594 goat anti-mouse IgM (Invitrogen). Co-staining images were taken using the ZEISS LS M 510 META confocal microscope.

Results

Detection and Isolation of ISFs in Mosquitoes Captured From the Northern Territory

A total of 4194 female mosquitoes as 94 pools were collected from various sites in the Northern Territory of Australia. Mosquitoes of five genera were assessed including Aedes (six species), Culex (seven species), Anopheles (two species), Coquillettidia (one species) and Mansonia (one species) (data not shown). Just under half (n = 2032) of the mosquitoes tested were mosquitoes yielding flaviviral isolates when inoculated onto C6/36 culture supernatant from inoculated C6/36 cells tested positive in RT-PCR. Of these RT-PCR-positive pools, only the Coquillettidia xanthogaster mosquitoes yielded flaviviral isolates when inoculated onto C6/36 cultures (Table 1). Partial NS5 gene sequencing revealed that each of these isolates has >98% nucleotide identity to the prototype isolate (isolate 56) and are likely to be a strain of the same virus, which we have tentatively named Palm Creek virus (PCV) (Table 2). Further analysis is being performed on the seven flavivirus isolation-negative pools. ISF-like sequences were amplified from two of these pools and these data will be published in a separate article.

Sequencing and Phylogeny of the Full Length Genome of PCV

The complete coding region of the PCV genome was sequenced (Genbank accession number KC505248). The PCV ORF encodes a polypeptide that consists of 3,364 amino acids and putative cleavage sites that are similar to those found in the polypeptides of other ISFs (data not shown, [6,14,37]). An optimised multiple sequence alignment for PCV and other ISFs over the NS5/3’ UTR and ORF regions was performed (Tables 2 and 3). The ORF alignment revealed that PCV is most closely related to the African ISF, NAKV, with 63.7% nucleotide identity (Table 3). Interestingly, the amino acid identity (66.6%) is only slightly higher than the nucleotide identity, suggesting that a large number of these changes are non-synonymous substitutions (Table 3). The next closest relatives of PCV over the entire coding region are QBV, CxFV and CTFV with nucleotide identities of 56.2%, 55.7–56.4% and 55.9–56% respectively. As expected, PCV was also closely related to NAKV over the NS5/3’UTR region with amino acid and nucleotide identities of 81% and 67.6% respectively.

Phylogenetic analysis of the nucleotide sequence of the NS5 and partial 3’ UTR regions of four PCV isolates revealed that these are highly similar and cluster with other ISFs, although the bootstrap support is low, and we were unable to conclude the location of PCV in the ISF group from this region alone (Fig. 1A). In contrast, bootstrap support for the complete genome tree is high and shows that PCV clusters most closely with NAKV virus, with 100% bootstrap support (Fig. 1B).

Growth of Viral Isolates in Different Cell Lines

The ability of PCV to infect a range of cells was assessed by regularly monitoring the cell monolayers for evidence of CPE and by assessing the cell monolayers for the presence of viral RNA by RT-PCR. While PCV replicated in C6/36 cells, as determined by a positive reaction in RT-PCR, no evidence of growth was observed in any of the vertebrate cells lines tested (Table 4). Although the first and second passage of each isolate showed no evidence of CPE in C6/36 cells, often by passage 4 there was clear evidence of morphological changes such as syncytia and vacuolation in cells infected with each isolate (Fig. S1B). It was also observed that fusion of the C6/36 cells by PCV could be enhanced

Table 1. Summary of PCV isolates from mosquito pools collected in Darwin.

| Pool No. | Date Collected | Mosquito Species      | Location          | RT-PCR* | Isolation# | Virus ID by sequence |
|---------|----------------|-----------------------|-------------------|---------|------------|----------------------|
| 22      | 13 Apr 2010    | Cq. xanthogaster       | Holmes Jungle, Darwin | +       | +          | Palm Creek virus (PCV) |
| 56      | 19 May 2010    | Cq. xanthogaster       | Palm Creek, Darwin | +       | +          | PCV                  |
| 73      | 8 Jun 2010     | Cq. xanthogaster       | Palm Creek, Darwin | +       | +          | PCV                  |
| 77      | 4 Jun 2010     | Cq. xanthogaster       | Berrimah Farm, Darwin | +       | +          | PCV                  |
| 90      | 4 Jun 2010     | Cq. xanthogaster       | Berrimah Farm, Darwin | +       | +          | PCV                  |
| 91      | 4 Jun 2010     | Cq. xanthogaster       | Berrimah Farm, Darwin | +       | +          | PCV                  |

*Mosquito homogenate tested positive in RT-PCR.
**Culture supernatant from inoculated C6/36 cells tested positive in RT-PCR.

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by reducing the pH of the cell culture medium to pH 6 (Fig. S1D and E). These observations are consistent with previous studies with this cell line and other flaviviruses [39].

Monoclonal Antibodies to PCV-Specific Antigens

Our initial analysis of the PCV isolates with a panel of mAbs that were pan-reactive to vertebrate-infecting flaviviruses (e.g. mAb 4G2 [40] and mAb 4G4 [30]), revealed a lack of recognition in a fixed-cell ELISA and Western blot (results not shown) and confirms that these viruses are antigenically distinct from other members of the flavivirus genus.

To obtain antibodies reactive to PCV antigens as research tools for further study of this virus and possibly other ISFs, hybridomas were produced to a preparation of concentrated PCV virions from infected mosquito cell culture supernatant. Three mAbs (3D6, 8G2, 9G4) that recognise PCV-specific antigens were obtained from the resulting hybridomas. These mAbs reacted with acetone-fixed PCV-infected cells by IFA and ELISA and failed to bind to uninfected cells in these assays (Fig. 3). As expected, no reaction was detected to cells infected with any of the vertebrate-infecting flaviviruses including WNV, KUNV, MVEV, yellow fever virus (YFV) and dengue virus (DENV), consistent with our earlier data (Table 5).

The three anti-PCV mAbs were all of the IgM isotype and recognised highly conformational epitopes that were sensitive to SDS-denaturation and hence, did not react with PCV antigens in Western blot (Table 5).

| Table 2. Amino acid and nucleotide identities between PCV and other ISFs over the NS5/3’UTR region. |
|---------------------------------------------------------------|
| **PCV56** | **PCV77** | **PCV22** | **PCV73** | **NAKV** | **CFAV** | **QBV** | **CTFV** | **Calbertado** | **AeFV** | **CxFV** | **KRV** |
| PCV56 | 100/100 | 100 | 100 | 100 | 81 | 57 | 74 | 74.5 | 59.5 | 60 | 70.5–73.5 | 62 |
| PCV77 | 99 | 100/100 | 100 | 100 | 100 | 81 | 57 | 74 | 74.5 | 59.5 | 60 | 70.5–73.5 | 62 |
| PCV22 | 98.8 | 99.6 | 100/100 | 100 | 100 | 81 | 57 | 74 | 74.5 | 59.5 | 60 | 70.5–73.5 | 62 |
| PCV73 | 99.1 | 99.8 | 99.5 | 100/100 | 100 | 81 | 57 | 74 | 74.5 | 59.5 | 60 | 70.5–73.5 | 62 |
| NAKV | 67.6 | 68.1 | 68.5 | 68 | 100/100 | 59–60 | 73.5 | 71.5 | 61.5 | 57.5 | 70–73.5 | 60.5 |
| CFAV | 55–56 | 55.5–56.5 | 55.3–56.3 | 55.5–56.5 | 58.8–59.6 | 95.5–100 | 55.5–56.5 | 55.5–56.5 | 54–55 | 54.5–56 | 55–56 | 82.5–83 |
| QBV | 66.6 | 66.6 | 66.8 | 66.5 | 66.1 | 54.8–55 | 100/100 | 85.5 | 59.5 | 57.5 | 83–86.5 | 58.5 |
| CTFV | 65 | 64.5 | 64.6 | 64.5 | 63.3 | 53.8–54.3 | 71.6 | 100/100 | 60.5 | 55.5 | 83.5–88 | 57.5 |
| Calbertado | 58.8 | 59 | 58.6 | 59.1 | 58.3 | 55.1–55.6 | 59.3 | 59.3 | 100/100 | 54.5 | 59–61 | 56.5 |
| AeFV | 57.1–57.6 | 56.6–57.1 | 56.6–57.1 | 56.8–57.1 | 56.0–68.3 | 55.5–55.6 | 56.8–57.1 | 53.3–53.5 | 100/100 | 54.5–56.5 | 81 |
| CxFV | 66.1–67.8 | 66.1–67.8 | 66.1–67.8 | 66.1–67.8 | 63.6–64.8 | 55.3–58.5 | 71.6–74 | 73–74.8 | 60.1–62.3 | 55.3–57.8 | 82.5–100 | 87.3–99.8 | 55.5–56.5 |
| KR V | 59.1 | 58.6 | 58.8 | 58.8 | 59.6 | 70–71.5 | 57.5 | 57 | 56.8 | 69.8–70.1 | 57.5–59 | 100/100 |

[Bold text: amino acid identity. Non bolded text: nucleotide identity.]

*A range is given for those viruses that have multiple isolates listed on Genbank.

PCV – Palm Creek virus; NAKV – Nakiwogo virus; CFAV – cell fusing agent virus; QBV – Quang Binh virus; CTFV – Culex theileri flavivirus; AeFV – Aedes flavivirus; CxFV – Culex flavivirus; KRV – Kamiti River virus.

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| Table 3. Amino acid and nucleotide identities between PCV and other ISFs over the entire coding region. |
|---------------------------------------------------------------|
| **PCV56** | **NAKV** | **CxFV** | **QBV** | **CTFV** | **AeFV** | **KRV** | **CFAV** |
| PCV56 | 100/100 | 66.6 | 54.5–54.4 | 54.5 | 53.8–53.9 | 37.6 | 37.9 | 41.9–42 |
| NAKV | 63.7 | 100/100 | 51.8–52.1 | 51.7 | 51.4–51.5 | 37.1 | 38 | 40.3 |
| CxFV | 55.7–56.4 | 54.5–54.8 | 96.3–100/90.1–100 | 69.3–67.7 | 70.7–71.2 | 37.9–38.1 | 38.2–38.4 | 45.3–45.5 |
| QBV | 56.2 | 54.7 | 65.8–66 | 100/100 | 73–73.1 | 38.3 | 37.6 | 45.5–45.7 |
| CTFV | 55.9–56 | 54.9 | 66.8–67 | 67.6–67.7 | 99.3–100/99–100 | 37.6 | 38.5 | 46.5–46.7 |
| AeFV | 44.8 | 44.5 | 45.5–45.8 | 45 | 45.3 | 100/100 | 68.3 | 59.6–60 |
| KR V | 45.3 | 45.7 | 45.8–46 | 45.9 | 46.2 | 64.6 | 100/100 | 65–65.5 |
| CFAV | 47.3–47.4 | 47.2–47.4 | 50–50.4 | 50.1 | 50.8 | 59.4–59.5 | 62.8–62.9 | 97.4–100/96–100 |

[Bold text: amino acid identity. Non bolded text: nucleotide identity.]

*A range is given for those viruses that have multiple isolates listed on Genbank.

PCV – Palm Creek virus; NAKV – Nakiwogo virus; CFAV – cell fusing agent virus; QBV – Quang Binh virus; CTFV – Culex theileri flavivirus; AeFV – Aedes flavivirus; CxFV – Culex flavivirus; KRV – Kamiti River virus.

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Recognition of Recombinantly Expressed PCV NS1 Protein by Anti-PCV mAbs

To assist in determining which PCV viral protein is recognised by each of the anti-PCV mAbs, three constructs were prepared for the expression of partial NS5 and the secreted expression of NS1, prM and E proteins. Each gene was cloned into a mammalian expression vector, upstream of the genes for V5 and HIS affinity tags. COS-7L cells were transiently transfected with each of these constructs.

Figure 1. Phylogenetic tree showing relationship between PCV and other insect-specific flaviviruses. (A) Maximum likelihood tree constructed using NS5/3’UTR sequences of select insect-specific flaviviruses and four isolates of PCV. (B) Maximum likelihood tree constructed using the complete ORFs of a selection of insect-specific flaviviruses. For both trees, the numbers at the nodes represent bootstrap replicates as a percentage of 1000 replicates. Both trees have been mid-point rooted. CFAV and CxFV groups have been collapsed for clarity.
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constructs and when the cells were fixed post-transfection, expression of recombinant protein was confirmed by the specific reaction of the anti-V5 antibody to the affinity tag fused to the expressed protein in IFA (Fig. 4A). Furthermore, two of the anti-PCV mAbs (3D6 and 9G4) specifically recognised the recombinant NS1 protein expressed in the transfected cells. The third anti-PCV mAb (8G2) did not recognise any of the recombinant proteins (data not shown).

Analysis of Recombinant PCV NS1 and E Proteins

To confirm whether the PCV E and NS1 proteins were glycosylated, the recombinantly expressed proteins were digested with PNGase F to remove N-linked glycans. Recombinant WNV NS1, which expresses three N-linked glycans, was similarly digested. Removal of the N-linked glycans is characterised by an increase in the mobility of the proteins and was seen for both PCV E and NS1 proteins to a similar extent as WNV NS1 (Fig. 4B). These data suggests that both PCV E and NS1 proteins are likely to utilize two to three glycosylation sites. This is consistent with the in silico predicted glycosylation sites for PCV NS1 and E proteins (two and three sites respectively; Net Nglyc 1.0 Server, www.cbs.dtu.dk/services/NetNGlyc).

Secretion of recombinant PCV NS1 protein was confirmed following the harvesting of the transfection cell culture supernatant and detection of the expressed protein in Western blot (Fig. 4C). While the NS1 monomer was clearly detected at a molecular weight of approximately 50 kDa, a fainter band with an apparent molecular weight of approximately 70 kDa was also visible. While it could be suggested that this larger band is an NS1 dimer, it is considerably smaller then the corresponding band for recombinant WNV NS1 dimer which is visible at approximately 100 kDa (Fig. 4D).

PCV Suppresses the Replication of Medically Significant Flaviviruses In Vitro

To determine whether prior infection of mosquito cells with ISFs could suppress subsequent infection with pathogenic, vertebrate-infecting flaviviruses, C6/36 cultures were infected with PCV (or they were mock-infected) 6–7 days prior to a secondary inoculation with the flaviviruses, MVEV or WNVKUNV. The infectious titres of the secondary infecting virus was compared to those produced in the PCV-negative controls. The results show that the infectious titres of the secondary infecting flaviviruses in study 1 were significantly reduced in PCV infected cells compared to PCV-negative cells at 24 hr (WNVKUNV 1.7 log reduction, \( p = 0.012 \); MVEV 1.63 log reduction, \( p = 0.0003 \) Student’s two-tailed t test) (Fig. 5B). A reduction in the infectious titre of WNVKUNV and MVEV in PCV-infected cells was similarly observed at 48 hr (Student’s two-tailed t test WNVKUNV \( p = 0.0032 \) (1 log), MVEV \( p = 0.0004 \) (1.63 log)) (Fig. 5C). This was consistent with the IFA images which showed dramatically reduced numbers of infected cells in the primary infected cultures compared to the mock-infected controls (Figs. 5A and D). In comparison, infection with the alphavirus RRV (which uses a different replication strategy) was permissive to the same level in both PCV-infected and uninfected cells (Student’s two-tailed t test

Table 4. Replication of PCV56 in various cell lines.

| Isolate   | Replication of Viral Isolates in various cell lines |
|-----------|---------------------------------------------------|
|           | C6/36    | BHK-21 | PS-EK | Vero | SW13 |
|           | CPE | RT-PCR | CPE | RT-PCR | CPE | RT-PCR | CPE | RT-PCR | CPE | RT-PCR |
| PCV56     | +   |        | -   | -    | -   | -     | -   | -     | -   | -     |
| WNVKUNV*  | +   | ND     | +   | ND   | +   | ND    | +   | ND    | +   | ND    |

*KUNV used as positive control in these experiments.
ND Not done.
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that mosquito cells persistently infected with an ISF are considerably less permissive to pathogenic flaviviruses 24–48 hr post-infection.

**Discussion**

The discovery of PCV in mosquito populations in Darwin represents the first isolation of an ISF in Australia. Our phylogenetic data also reveal a close relationship between PCV and other ISFs, placing them in the same clade as the prototype ISF (CFAV) and most of the more recent ISF isolates from around the world [4,6,8–10,14,23,41]. Isolation of PCV from *Coquillettidia xanthogaster* also represents the first isolation of an ISF from a member of the *Coquillettidia* genus.

Presently, the distribution of PCV and its insect host restriction in Australia is not clear; however a preliminary study of mosquitoes trapped in the Kimberley region of north-western Australia have detected PCV-like viruses in both *Coquillettidia* and *Culex* species suggesting the virus could be more widespread and present in additional mosquito species (Nguyen, McLean, Hobson-Peters, Barnard, Johansen and Hall, unpublished data). Indeed, in this present study, PCV was isolated from 33.3% of the *Cq.* xanthogaster pools assessed and suggests a high prevalence of this ISF in *Coquillettidia* mosquito populations within the Northern Territory of Australia.

Our phylogenetic data suggests that PCV is most closely related to NAKV, an ISF isolated from *Mansonia* species in Uganda. However, these viruses share only 63.7% nucleotide identity over the ORF, which clearly indicates that PCV represents a new species [22] and that PCV and NAKV have clearly evolved separately for some time. It is worth noting that *Mansonia* mosquitoes are closely related to *Coquillettidia*, both belonging to the Mansoniini mosquito tribe [42]. This is suggestive of co-evolution of these viruses with their mosquito hosts. However this hypothesis is not supported by the work of Cook et al. (2012) where no significant proof for the co-divergence of insect-specific flaviviruses and their vectors was identified [43].

Our *in vitro* findings that mosquito cells previously infected with PCV were less permissive to subsequent infection with WNV and MVEV, in a flavivirus-specific manner, provide evidence that ISFs may down-regulate the replication of vertebrate-infecting flaviviruses in mosquito tissues. However, *in vivo* studies are yet to be performed and it must be noted that the response to flaviviral infection *in vivo* may differ to that seen in C6/36 cells due to the defective innate immune response of these cells [44,45]. Despite this, several *in vitro* studies have shown that prior infection of cells with one flavivirus can inhibit replication with a related flavivirus subsequently inoculated into the culture [46–48]. This phenomenon is known as superinfection exclusion and has been postulated as a mode of competition for mosquito hosts between related flaviviruses.

### Table 5. Characterisation of anti-PCV mAbs.

| mAb ID | Isotype | Protein specificity | Reactivity in WB | Virus Specificity* |
|--------|---------|---------------------|------------------|-------------------|
|        |         |                     |                  | PCV               | WNV | MVEV | DENV | YFV |
| 3D6    | IgM/K   | NS1                 | –                | +                 | –   | –    | –    | –  |
| 8G2    | IgM/K   | ?                   | –                | –                 | –   | –    | –    | –  |
| 9G4    | IgM/K   | NS1                 | –                | +                 | –   | –    | –    | –  |

*Binding pattern of mAbs in fixed cell ELISA to PCV, Palm Creek virus; WNV, West Nile virus Kunjin subtype; MVEV, Murray Valley encephalitis virus; DENV, Dengue virus; YFV, yellow fever virus.

**WB** = Western blot.
viruses. The molecular mechanisms of superinfection exclusion are thought to involve competition for, or modification of cellular factors that result in reduced receptor binding, viral entry or RNA replication [49]. Indeed, recent studies by Zou et al. (2009) using WNV and mammalian cells (BHK-21), identified a mechanism of superinfection exclusion between flaviviruses at the step of viral RNA synthesis, reportedly due to the sequestering of cellular factors (host proteins and membranes) to support RNA replication of the primary infecting virus, thereby depriving the second infecting virus of essential elements of replication [50].

Interactions between CxFV and WNV have been assessed in vitro by other groups. In one similar experiment in C6/36 cells, primary infection with CxFv and subsequent infection with WNV 48 hr later resulted in significant differences in the WNV titres, but not until 84–156 hr post-infection [17]. This contrasts our data where we observed differences in the titres of WNV_KUNV and MVEV at 24 and 48 hr post-infection. In another study, C6/36 cells sequentially infected with CxFv and WNV also resulted in lower titres of infectious WNV secreted from cells infected with CxFv compared with those that were mock infected. However, these differences were not statistically significant and death of the cells infected with CxFv was also observed [18].

The high prevalence of ISFs in some populations of mosquitoes has prompted speculation on the role of ISFs in regulation of the transmission of pathogenic flavivirus by superinfection exclusion or similar mechanism. However, recent studies report that co-infection of Cx. quinquefasciatus mosquitoes from Honduras with an ISF from Guatemala (CxFV Izabal) and WNV significantly enhanced the transmission rate of the latter [18]. This was consistent with reports by Newman et al. (2011) of a positive ecological association between CxFV and WNV in co-infected mosquito pools collected in the field, whereby pools of Cx. pipiens that were positive for WNV, were four times more likely to also be positive for CxFV [51]. Competitive interaction between CxFV and WNV in vitro has also been observed by Bolling et al. (2012) [17]. In this vector competence study using Cx. pipiens mosquitoes, early suppression of WNV replication and dissemination was seen in mosquitoes persistently infected with CxFV. While the mechanism is not yet understood, these preliminary studies provide the first evidence that ISFs may affect the transmission of pathogenic mosquito-borne viruses in nature. However, the large genetic diversity between ISFs identified in different parts of the world and our own data showing an inhibitory effect of PCV on the replication of other Australian flaviviruses in vitro, indicates that this relationship may vary with the species of virus (and mosquito) under examination. In vivo experiments are currently underway to determine whether prior infection of Australian mosquito species with PCV can also inhibit or delay the transmission of WNV_KUNV.

The derivation of three hybridomas reactive to PCV antigens in this study is the first report of mAbs specific for an ISF. The lack of growth of these viruses in vertebrate cells and the absence of
consistent and clearly observable CPE that they produce in mosquito cells, particularly at low passage, has to date required the use of RT-PCR to detect and monitor the growth of these viruses in mosquitoes and cell culture. Two of the mAbs were shown to be reactive to the PCV NS1 protein. Since the epitope recognised by these mAbs is sensitive to SDS denaturation, it is possible that these mAbs specifically bind a larger NS1 dimeric or hexameric complex [32] and would be consistent with the method of immunogen concentration from cell culture supernatant using large molecular weight cut off concentrators. The protein bound by the third mAb (6G2) could not be determined. The epitope bound by this mAb may not be authentically expressed on the recombinant proteins, or, this mAb may bind E or prM/E complexes. Although recombinant prM/E was assessed in this study, it should be noted that the lack of the E protein transmembrane domain, as well as the presence of the C-terminal V5/HIS tag is likely to have prevented sub-viral particle (SVP) assembly. A construct for the expression of PCV SVPs is currently being made.

The three mAbs appear to be specific for PCV as no cross-reactivity was seen to any of the vertebrate-infecting flaviviruses assessed and our preliminary data suggests that there is negligible or no reactivity to other ISFs. This provides evidence that PCV is antigenically distinct from other closely related ISFs, although further assessment of these mAbs with PCV’s closest relative, NAKV, is required.

Digestion of the recombinant PCV NS1 and E proteins with PNGase F revealed that both of these proteins possess two or three N-linked glycans. While the E protein of the vertebrate-infecting flaviviruses, such as WNV and MVEV is normally singly glycosylated, or not glycosylated at all [35,53–55], the insect-specific flaviviruses QBV and KRV contain 6 potential E glycosylation sites, although only 2–3 are utilised [4,9].

In summary, we provide the first isolation of an ISF from mosquitoes collected in Australia. Additionally, we report the first ISF-specific mAbs. The advent of these new reagents will allow rapid detection of PCV in vitro and in vivo and will be useful tool for further research on the biology of these viruses. A thorough investigation on the ecology and epidemiology of this virus and of similar viruses circulating in Australia will provide valuable insight into the biological relevance of this important group of viruses.

Supporting Information

Figure S1 Phase contrast microscopy of C6/36 cells infected with PCV at pH 6 and pH 7. Mock (A) and PCV-infected cells (B) four days post-infection with virus at passage 4 under standard culturing conditions. Fusion of the PCV-infected cells was enhanced by reducing the culture medium pH to 6: (C) Uninfected C6/36 cells (∼200) in pH 6 medium; (D) PCV-infected cells (∼200) in pH 6 medium; (E) PCV-infected cells (∼400) in pH 6 medium; (F) PCV-infected cells (∼400) in pH 7 medium. (TIF)

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Author Contributions

Conceived and designed the experiments: JHP YXS FJM NAP SW NH LM PW BJB RAH. Performed the experiments: JHP AWYY JWFL YXS FJM NAP RJ RH RAH. Analyzed the data: JHP AWYY FJM NAP BJB RAH. Contributed reagents/materials/analysis tools: NK SW SD MW.

References

1. Mackenzie JS, Lindsay MD, Coelen RJ, Broom AK, Hall RA, et al. (1994) Arboviruses causing human disease in the Australasian zoogeographic region. Arch Virol 136: 447–467.
2. Mackenzie JS, Gubler DJ, Petersen LR (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10: 890–109.
3. Stollar V, Thomas VI (1975) An agent in the Aedes aegypti cell line (Peleg) which causes fusion of Aedes albopictus cells. Virology 64: 367–374.
4. Crabtree MB, Sang RC, Stollar V, Dunster LM, Miller BR (2005) Genetic and phenotypic characterization of the newly described insect flavivirus, Kamiti River virus. Arch Virol 149: 1055–1110.
5. Cook S, Bennett SN, Holmes EC, De Cleose R, Moureau G, et al. (2006) Isolation of a novel strain of the flavivirus cell fusing agent virus in a natural mosquito population from Puerto Rico. J Gen Virol 87: 733–748.
6. Hoshino K, Iwata H, Tsuda Y, Yano K, Sasaki T, et al. (2007) Genetic characterization of a new insect flavivirus isolated from Culex pipiens mosquito in Japan. Virology 359: 405–414.
7. Hoshino K, Iwata H, Tsuda Y, Sotome K, Kobayashi M (2009) Isolation and characterization of a new insect flavivirus from Aedes albopictus and Aedes flavipictus mosquitoes in Japan. Virology 391: 119–129.
8. Calzolari M, Ze-Ze L, Ruzek D, Vazquez A, Jeffries C, et al. (2012) Detection of mosquito-only flaviviruses in Europe. J Gen Virol 93: 1215–1223.
9. Crabtree MB, Ng A-P, Miller BR (2009) Isolation and characterization of a new mosquito flavivirus, Quang Binh virus, from Vietnam. Arch Virol 154: 857–860.
10. Cook S, Moureau G, Harbach RE, Mukwaya L, Goodger K, et al. (2009) Isolation of a novel species of flavivirus and a new strain of Culex flavivirus (Flaviviridae) from a natural mosquito population in Uganda. J Gen Virol 90: 2669–2678.
11. Huhtamo E, Parkuri N, Kurkela S, Manni T, Valeri A, et al. (2009) Characterization of a novel flavivirus from mosquitoes in northern Europe that is related to mosquito-borne flaviviruses of the tropics. J Virol 83: 9532–9540.
12. Jungel S, Kepp A, Kurth A, Psil G, Ellebroek H, et al. (2009) A new flavivirus and a new vector: characterization of a novel flavivirus isolated from uranotaenia mosquitoes from a tropical rain forest. J Virol 83: 4462–4468.
13. Bolling BG, Eisen L, Moore CG, Blair CD (2011) Insect-specific flaviviruses from Culex mosquitoes in Colorado, with evidence of vertical transmission. Am J Trop Med Hyg 85: 169–177.
14. Parreira R, Cook S, Lopes A, de Matos AP, de Almeida AP, et al. (2012) Genetic characterization of an insect-specific flavivirus isolated from Culex theileri mosquitoes collected in southern Portugal. Virus Res 167: 152–161.
15. Lutomiah JJ, Mwandawiro C, Magambo J, Sang RC (2007) Infection and vertical transmission of Kamiti river virus in laboratory bred Aedes aegypti mosquitoes. J Insect Sci 7: 1–7.
16. Sajyasombat R, Bolling BG, Braul AC, Bartholomay LC, Blitvich BJ (2011) Evidence of efficient transovarial transmission of Culex flavivirus by Culex pipiens (Diptera: Culicidae). J Med Entomol 48: 1031–1038.
17. Bolling BG, Olfar-Popelka EJ, Eisen L, Moore CG, Blair CD (2012) Transmission dynamics of an insect-specific flavivirus in a naturally infected Culex pipiens laboratory colony and effects of co-infection on vector competence for West Nile virus. Virology 427: 90–97.
36. Broom AK, Hall RA, Johansen CA, Oliveira N, Howard MA, et al. (1998) Identification of monoclonal antibodies in ELISA. Pathology 30: 286–288.

35. Adams SC, Broom AK, Sammels LM, Hartnett AC, Howard MJ, et al. (1995) Biological, antigenic and phylogenetic characterization of the flavivirus Allfin. J Gen Virol 87: 329–337.

34. Cook S, Moreau G, Kinchen A, Gould EA, de Lamballerie X, et al. (2012) Molecular evolution of the insect-specific flaviviruses. J Gen Virol 93: 223–234.

33. Davis BS, Chang GJ, Cropp B, Roehrig JT, Martin DA, et al. (2001) West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge. J Med Virol 63: 323–337.

32. Chang GJ, Hunt AR, Davis B (2000) A single intramuscular injection of CFA and the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189: 311–324.

31. Hobson-Peters J, Toye P, Sanchez MD, Bossart KN, Wang LF, et al. (2008) A plasmid expressing the nonstructural protein NS1 from WNV Kunjin strain, Australia, 2011. Emerg Infect Dis 18: 304–307.

30. Clark DC, Lobigs M, Lee E, Howard MJ, Clark K, et al. (2007) In vitro reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein. J Gen Virol 88: 1175–1183.

29. Felsenstein J (1989) PHYLIP–Phylogeny Inference Package (Version 3.2). Cladistics 5: 164–166.

28. May FJ, Lobigs M, Lee E, Gendle DJ, Mackenzie JS, et al. (2006) Biological, antigenic and phylogenetic characterization of the flavivirus Allfin. J Gen Virol 87: 329–337.

27. Gorman BM, Leer JR, Filippich C, Goss PD, Doherty RL (1975) Plaquing and neutralisation of arboviruses in the PS-EK line of cells. Australian Journal of Medical Thechnology 6: 65–71.

26. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

25. Felsenstein J (1989) PHYLIP–Phylogeny Inference Package (Version 3.2). Cladistics 5: 164–166.

24. Blitvich BJ, Lin M, Dorman KS, Soto V, Hovav E, et al. (2009) Genomic sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189: 311–324.

23. Blitvich BJ, Lin M, Dorman KS, Soto V, Hovav E, et al. (2009) Genomic sequence of cell fusing agent (CFA): homology between the nonstructural proteins encoded by CFA and the nonstructural proteins encoded by arthropod-borne flaviviruses. Virology 189: 311–324.

22. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52: 696–704.

21. Ritchie SA, Phillips D, Broom A, Mackenzie J, Poidinger M, et al. (1997) Isolation of Japanese encephalitis virus from Culex tarsalis Mosquitoes from western Canada with viruses isolated in California and Colorado. Am J Trop Med Hyg 56: 80–84.

20. Whelan PL, Weir RP (1993) The isolation of alpha and flavi viruses from mosquitoes in the Northern Territory 1982–1992. Arbovirus Research in Australia 6: 270–278.

19. Rohe D, Fall R (1979) A miniature battery powered CO2 housed light trap for mosquito-borne encephalitis surveillance. Bulletin of the society of vector ecology 4: 24–27.

18. Kent RJ, Cribb RB. Miller BR (2010) Transmission of West Nile virus by Culex quinquefasciatus say infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4: e671.

17. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB (1998) Phylogeny of the genus Flavivirus. J Virol 72: 73–83.

16. St John N, McInerney O, Stocker R, Dinesh M, Ireland A, et al. (2000) The isolation of alpha and flavi viruses from mosquitoes in the Northern Territory 1982–1992. Arbovirus Research in Australia 6: 270–278.

15. Adams SC, Broom AK, Sammels LM, Hartnett AC, Howard MJ, et al. (1995) Identification of monoclonal antibodies in ELISA. Pathology 30: 286–288.

14. Gentry MK, Henschel EA, McCoson JM, Brandt WE, Daleyville JM (1992) Identification of distinct antigenic determinants on dengue-2 virus by monoclonal antibodies. Am J Trop Med Hyg 53: 548–555.

13. Tyler S, Bolling BG, Blair CJ, Brandt AC, Pabbaraju K, et al. (2011) Distribution and phylogenetic comparisons of a novel flavivirus sequence present in Culex tarsalis Mosquitoes from western Canada with viruses isolated in California and Colorado. Am J Trop Med Hyg 85: 162–168.

12. Harbach RE, Kitching IJ (1998) Phylogeny and Classification of the Culicidae (Diptera). Systematic Entomology 23: 327–370.

11. Rose D, Fall R (1979) A miniature battery powered CO2 housed light trap for mosquito-borne encephalitis surveillance. Bulletin of the society of vector ecology 4: 24–27.

10. Kent RJ, Cribb RB, Miller BR (2010) Transmission of West Nile virus by Culex quinquefasciatus say infected with Culex Flavivirus Izabal. PLoS Negl Trop Dis 4: e671.