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 theileri flavivirus (CTFV [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 cycle. In 1975, Stollar and Thomas reported...
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) [18] and Bolling et al. (2012) [17] suggesting that coinfection 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 samples 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 sites around the Northern Territory of Health conducts regular mosquito monitoring and has done so for many years. These field studies did not involve endangered or protected species.

Viral RNA Detection and Isolation from Mosquito Homogenates

Mosquito homogenates were screened for the presence of flaviviral 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 RNeasy 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 30°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 harvesting. 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.
Immunofluorescence Assay (IFA)

Antibodies to Palm Creek Virus (PCV) were produced and characterized using a method previously described [29]. Briefly, PCV-infected C6/36 cell culture supernatants were concentrated through a high molecular weight cut-off (300 K) centrifugal concentrator (Sartorius). Concentrated samples were placed onto a cover glass and allowed to incubate for 4–5 days. The cells were seeded into 24-well plates overnight (o/n). Transfection was performed using Lipofectamine 2000 (Invitrogen) following manufacturer’s instructions. After 21 hr, cells were fixed onto cover slips by soaking in ice cold 100% acetone for 5 min. IFA was performed as described above, however, in this assay, blocking was performed using a blocking buffer comprised of 0.05 M Tris–HCl pH 8.0, 1 mM EDTA, 0.1% Tween 20 and 0.2% w/v casein and the washing was performed with PBS containing 0.05% tween-20 (PBS/T). The bound anti-PCV mAbs were detected with Alexa Fluor 594 goat anti-mouse IgM (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) glycoproteins were amplified by RT-PCR and cloned into mammalian expression vectors pCMV55-mSyn (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 recombinant gene fragment was authentic and in frame for correct translation.

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 supernatant 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 Titer-Max Gold adjuvant (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 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 published methods [30]. However, in this case, incubation steps were performed at 37°C to enhance sensitivity of the assay. The hybridomas were cloned by limited dilution and harvested culture supernatants stored under sterile conditions at 4°C until used.

PCV-reactive monoclonal antibodies (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 as described previously, except that an incubation temperature of 37°C was used [30,31]. The isotype of each mAb was determined using the Mouse typer kit (Bio-Rad) according to the manufacturer’s instructions.

Immunofluorescence Assay (IFA)

C6/36 cells were grown on glass coverslips and infected with PCV or WNV (Kunjin virus MRMI6; WNV-KUNV), 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 FU2/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 cover glass, negatively stained with 1% uranyl acetate and viewed in a JEOL1010 transmission electron microscope.

Analysis of Virus Replication in Mammalian Cell Lines

Stocks of PCV (isolate 56) and WNV (Kunjin virus MRMI6; WNV-KUNV), 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 FU2/cFD3 primer pair. Use of the PCK V5 antibody found the following: any changes in protein mobility as previously described [35].

Superinfection Exclusion Experiments

Monolayers of C6/36 cells were inoculated at 80% confluence 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 MR16 subtype – WNV KUNV) 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, WNV KUNV or RRV was examined by IFA staining using the flavivirus pan-reactive anti-NS1 mAb 4G4 [30] or RRV-specific mAb 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 480-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 LSM 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 *Culex* species, under half (n = 2032) of the mosquitoes tested were *Culex* species, and shows that PCV clusters most closely with NAKV virus, with In contrast, bootstrap support for the complete genome tree is high location of PCV in the ISF group from this region alone (Fig. 1A). In addition, 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).

**TEM Analysis of Virus Particle Morphology**

When concentrated culture supernatants from PCV-infected C6/36 cell cultures were examined under TEM, small spherical particles about 40–50 nm in size were observed (Fig. 2), consistent with the expected size of flavivirus particles [38].

**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 assaying 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 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. 5IB). It was also observed that fusion of the C6/36 cells by PCV could be enhanced

**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 polyprotein that consists of 3,364 amino acids and putative cleavage sites that are similar to those found in the polyproteins 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 most 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 ISF isolates revealed that these isolates 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).

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

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

* 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 [39], 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).
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.

doi:10.1371/journal.pone.0056534.g001
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 suggest 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 than 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 WNV<sub>KUNV</sub>. 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 (WNV<sub>KUNV</sub> 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 WNV<sub>KUNV</sub> and MVEV in PCV-infected cells was similarly observed at 48 hr (Student’s two-tailed \( t \) test WNV<sub>KUNV</sub> \( p = 0.0032 \) (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.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>C6/36</th>
<th>BHK-21</th>
<th>PS-EK</th>
<th>Vero</th>
<th>SW13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPE</td>
<td>RT-PCR</td>
<td>CPE</td>
<td>RT-PCR</td>
<td>CPE</td>
</tr>
<tr>
<td>PCV56</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>WNV&lt;sub&gt;KUNV&lt;/sub&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>+</td>
</tr>
</tbody>
</table>

*KUNV used as positive control in these experiments.
ND Not done.

doi:10.1371/journal.pone.0056534.t004

Figure 2. Transmission Electron Micrograph of PCV particles. Culture supernatant from C6/36 cells infected with PCV was concentrated and round, enveloped virus particles with an electron-dense core were visualised using uranyl acetate staining. Scale bars are 100 nm and 50 nm for left and right panels respectively.

doi:10.1371/journal.pone.0056534.g002
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 ISFs 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 in vitro findings that mosquito cells previously infected with PCV were less permissive to subsequent infection with WNVKUNV 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 ISFs.

<table>
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<th>Table 5. Characterisation of anti-PCV mAbs.</th>
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<td><strong>mAb ID</strong></td>
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</tr>
<tr>
<td>3D6</td>
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<td>8G2</td>
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<td>9G4</td>
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*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.
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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 WNVKUNV 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 WNVKUNV.

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 antigenetically 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. Analyzed the data: JHP AWYY FJM NAP BJR RAH. Contributed reagents/materials/analysis tools: NK SW SD RW LM NH PW. Wrote the paper: JHP FJM BJR RAH.

References