We describe the isolation and characterization of a novel flavivirus, isolated from a pool of *Culex (Melanoconion) ocosa* Dyar and Knab mosquitoes collected in 2009 in an urban area of the Amazon basin city of Iquitos, Peru. Flavivirus infection was detected by indirect immunofluorescent assay of inoculated C6/36 cells using polyclonal flavivirus antibodies (St. Louis encephalitis virus, yellow fever virus and dengue virus type 1) and confirmed by RT-PCR. Based on partial sequencing of the E and NS5 gene regions, the virus isolate was most closely related to the mosquito-borne flaviviruses but divergent from known species, with less than 45 and 71% pairwise amino acid identity in the E and NS5 gene products, respectively. Phylogenetic analysis of E and NS5 amino acid sequences demonstrated that this flavivirus grouped with mosquito-borne flaviviruses, forming a clade with Nounané virus (NOUV). Like NOUV, no replication was detected in a variety of mammalian cells (Vero-76, Vero-E6, BHK, LLCMK, MDCK, A549 and RD) or in intracerebrally inoculated newborn mice. We tentatively designate this genetically distinct flavivirus as representing a novel species, Nanay virus, after the river near where it was first detected.

**INTRODUCTION**

The genus *Flavivirus* (family *Flaviviridae*) comprises, at the time of writing, approximately 70 virus species, although there are probably many more species yet undiscovered (Pybus *et al.*, 2002). Flaviviruses are small, enveloped viruses with an approximately 11 kbp positive-sense RNA genome that encodes three structural proteins [capsid, premembrane/membrane and envelope (E)] and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), which play important roles in replication, proteolysis and maturation in infected cells (Mukhopadhuy *et al.*, 2005). Flaviviruses can be categorized based on their capacity to replicate in arthropod and vertebrate hosts, described as insect-specific, mosquito-borne, tick-borne and no known vector flaviviruses (Kuno *et al.*, 1998). These groupings are well supported by phylogenetic comparison of their RNA genomes and by their replication range in cell culture (Kuno, 2007; Kuno *et al.*, 1998).

Mosquito-borne flaviviruses and tick-borne flaviviruses, including yellow fever virus (YFV), dengue virus serotypes 1–4 (DENV-1 to DENV-4), Japanese encephalitis virus (JEV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV), are often associated with human infections and are the cause of high morbidity and mortality worldwide. In contrast, flaviviruses such as *Culex* flavivirus (CxFV), Kamiti River virus (KRV) and cell fusing agent virus (CFAV) have only been isolated from mosquitoes or mosquito cell lines, have no recognized association with vertebrates, and have not yet been associated with human or animal disease. These insect-specific flaviviruses have broad distribution both geographically (Blitvich *et al.*, 2009; Calzolari *et al.*, 2012; Cook *et al.*, 2006, 2009; Machado *et al.*, 2012) and in terms of host mosquito species (Cook *et al.*, 2012). While not responsible for human disease, these viruses are of increasing interest owing to possible interactions in the mosquito host with...
pathogenic flaviviruses and for advancing understanding of flavivirus evolution (Bolling et al., 2012; Kent et al., 2010; Newman et al., 2011).

The Amazon basin of South America is fertile ground for flaviviruses, with extensive circulation of DENV serotypes in urban areas, enzootic transmission of YFV (Auguste et al., 2010) and sporadic human illness caused by a variety of Culex-associated flaviviruses, including St. Louis encephalitis virus (SLEV), Bussuquara virus (BSQV) and Rocio virus (ROCV). Recently, insect-specific flaviviruses (CxFV) have also been reported in South America (Kim et al., 2009; Machado et al., 2012). To understand better the diversity of circulating arthropod-borne viruses, including flaviviruses, near human populations in the Amazon basin, we conducted a mosquito survey in urban zones of Iquitos, Peru. Here we describe the characterization of a novel flavivirus, isolated from Culex (Melanoconion) ocossa Dyar and Knab mosquitoes, with an apparent barrier to replication in mammalian cells.

RESULTS

A monolayer of C6/36 cells inoculated with a pool of females of Culex (Mel.) ocossa Dyar and Knab (n=12; collected in July 2009 from the Bellavista-Nanay neighbourhood of Iquitos) was reactive to DENV, YFV and SLEV polyclonal antibodies by immunofluorescence assay (IFA), indicating the presence of a flavivirus. No reaction was observed using DENV serotype-specific and YFV monoclonal antibodies. Attempts to culture the virus in mammalian cells, including simian (LLCMK, Vero-76 and Vero-E6), hamster (BHK), canine (MDCK) and human (RD and A549) cells were unsuccessful, as no cytopathic effects were observed and all cultures were negative by IFA and RT-PCR after multiple passages. Additionally, newborn mice inoculated intracerebrally with viral supernatants did not show signs of disease and no viral RNA could be detected by RT-PCR.

Two cDNA products were generated for molecular characterization: an 876 bp (292 aa) partial sequence of the E gene (covering base pairs 724–2346 of the full-length YFV 17D) and a 1032 bp (344 aa) partial sequence of the NS5 gene (covering base pairs 8993–10077 of full-length YFV 17D). Based on BLASTN search results (NCBI), the generated sequences belonged to the genus Flavivirus yet were highly divergent from the aligned regions in other flaviviruses. Based on uncorrected p-distances, the highest amino acid sequence identities were observed with mosquito-borne flaviviruses, at approximately 70 and 42% pairwise identity for the NS5 and E proteins, respectively. Given the high divergence, we tentatively identify this isolate as representing Nanay virus (NANV), after the Amazon River tributary near the location of the mosquito trap. Despite the low amino acid sequence identity, there were several conserved features in the sequenced regions of the E and NS5 genes. The E gene product contained the DTGHGT and PPXGXS motifs in domain III conserved across Culex-borne flaviviruses and mosquito-borne flaviviruses, respectively (Fig. 1). The E region contained the four disulfide bond-forming cysteine residues conserved among flaviviruses (Grard et al., 2010).

To explore further the genetic relationships among flaviviruses, phylogenetic analysis was conducted based on the partial E and NS5 genes and gene products. The overall tree topology was similar in the E and NS5 phylogenies (Fig. 2) and was robust to the different alignment and phylogenetic reconstruction approaches (data not shown). The resulting branching patterns were broadly similar to those previously described (Cook et al., 2012; Gaunt et al., 2001; Grard et al., 2010; Kuno et al., 1998) and consistent with International Committee on Taxonomy of Viruses groupings (http://ictvdb.bio-mirror.cn/ictv/fs_flavi.htm). The branching pattern for NANV was similar for all phylogenetic approaches, with NANV placed clearly within the mosquito-borne flavivirus group and distinct from the insect-only flaviviruses (Fig. 2). NANV did not cluster with the YFV/Entebbe bat virus (ENTV) group, the Aedes-borne flaviviruses or the Culex-borne flaviviruses. Instead in most analyses, including Bayesian and maximum-likelihood (ML) reconstructions based on E and NS5, NANV formed a group with Nounane virus (NOUV), albeit with low posterior probability or bootstrap support in some instances. Phylogenies based on Gblocks-stripped NS5 amino acid sequences and neighbour-joining (NJ) analysis of NS5 nucleotide sequences did not result in a monophyletic pattern for NANV and NOUV, indicating some uncertainty in the grouping.

DISCUSSION

We report the identification and characterization of a novel flavivirus isolated from Culex (Melanoconion) ocossa Dyar and Knab mosquitoes from Iquitos, Peru. Although there was some ambiguity in the phylogenetic placement of NANV in relation to another recently identified flavivirus (NOUV), overall it is clear that NANV is related most closely to mosquito-borne flaviviruses, yet distinct from the YFV/ENTV, Culex-borne and Aedes-borne flavivirus groups. Considering the high sequence divergence and the different topologies observed for different gene regions (Billoir et al., 2000; Cook & Holmes, 2006), the observed ambiguity in the branching patterns is not surprising. Yet, the placement of NANV in the phylogeny with respect to YFV/ENTV and the other mosquito-borne flavivirus groups was robust to all different model assumptions tested. Kuno et al. (1998) proposed criteria for demarcation of a species, defining a species as a class of virus with greater than 84% nucleotide sequence identity. As NANV has less than 67% nucleotide identity and less than 71% amino acid identity in pairwise comparison with other flaviviruses in a more conserved region (NS5 gene), NANV does not fall within existing flavivirus species and may
represent the first member of a novel species within the genus Flavivirus (Billoir et al., 2000; Kuno et al., 1998).

Despite the clear placement of NANV with mosquito-borne flaviviruses, all of which have been associated with infections of mammals and other vertebrates (Kuno, 2007), we did not detect replication of NANV in mammalian cells. Two other flaviviruses have been identified recently which share a similar genetic association with mosquito-borne flaviviruses and replication restriction in mammalian cells: NOUV, isolated from Uranotaenia mashonaensis in Côte d’Ivoire (Junglen et al., 2009), and Lammi virus (LAMV), isolated from Aedes cinereus in Finland (Huhtamo et al., 2009). Previously, flaviviruses that replicated only in insect cells, including KRV, CxFV and CFAV (Bliotvich et al., 2009; Cook et al., 2006, 2009, 2012; Hoshino et al., 2007), were restricted to a single genetic group, yet NANV, NOUV and LAMV are all clearly genetically distinct from the insect-only flaviviruses. In our study, NANV and NOUV were found to be sister taxa, forming a clade separate from other mosquito-borne flaviviruses. Also, in contrast to previous reports (Huhtamo et al., 2009), NOUV (and thus NANV) did not segregate with LAMV. One limitation of our study was the absence of complete ORF data for NANV, which could explain the discrepancy (Billoir et al., 2000). Alternatively, the discrepancy could be due to their use of a different genetic algorithm (Kimura two-parameter), which may be too simplistic for highly divergent data (Bos & Posada, 2005). Our preliminary ML and NJ analysis of available complete ORFs (therefore excluding NANV) suggested that more complex approaches (such as ML and more parameter-rich models) did not support a monophyletic pattern for LAMV and NOUV (data not shown).

As NANV did not replicate in mammalian cells, it is not clear if its persistence involves a vertebrate reservoir or is limited to vertical transmission in mosquitoes (Bolling et al., 2011). Tick-borne and mosquito-borne flaviviruses that are associated with vertebrate infections readily...

### Table: Principal Vector Clade

<table>
<thead>
<tr>
<th>Vector</th>
<th>Virus (accession no.)</th>
<th>E protein aa 310 – 319</th>
<th>aa 369 – 374</th>
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<td><strong>Cul. (Mel.) ocossa</strong></td>
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<td></td>
<td>SLEV (EU566860)</td>
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<td>P P F G A S</td>
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<td></td>
<td>CPCV (AF372417)</td>
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<td></td>
<td>IGUV (AY632538)</td>
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<td></td>
<td>BSVQ (CN009026)</td>
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<td></td>
<td>NJLV (AF372411)</td>
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<td>ARGV (AF372413)</td>
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<td>IHV (CN009028)</td>
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<td>ROCV (AY632542)</td>
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<td>KOKV (CN009029)</td>
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<tr>
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<td>BOUV (DO859057)</td>
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<td>ENTVC (CN008718)</td>
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<tr>
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<td>RBV (AF144692)</td>
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**Fig. 1.** Alignment of domain III amino acid sequences showing common motifs between NANV and other flaviviruses. Amino acid numbers (310–319 and 369–374) correspond to the E gene product of YFV 17D (GenBank accession no. X03700). Other viruses shown are St. Louis encephalitis virus (SLEV), Cacicapre virus (CPCV), Igaupe virus (IGUV), Bussuquara virus (BSVQ), Naranjal virus (NJV), Arora virus (ARAV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Ilheus virus (ILHV), Rocio virus (ROCV), Kokobera virus (KOKV), yellow fever virus vaccine strain 17D (YFV 17D), Boubou virus (BOUV), Edge Hill virus (EHV), Sepik virus (SEPV), dengue virus type 1 (DENV-1), dengue virus type 2 (DENV-2), dengue virus type 3 (DENV-3), dengue virus type 4 (DENV-4), Kidogou virus (KDEV), Zika virus (ZIKV), Spondweni virus (SPOV), Nounané virus (NOUV), Tyuleniy virus (TYUV), Meaban virus (MEAV), Kyasanur forest disease virus (KFDV), Omsk haemorrhagic fever virus (OHFV), Yokose virus (YOKV), Entebbe bat virus (ENTV) and Rio Bravo virus (RBV).
replicate in mammalian cell culture, whereas insect-specific flaviviruses do not (Kuno, 2007), suggesting that the absence of NANV in mammalian cells reflects an inability to infect vertebrates in nature. However, cultured cells may not be a perfect proxy for infection in vivo, replication may have been restricted by the elevated temperature of mammalian cell culture, or there may be a narrow vertebrate host range not reflected in our selection of mammalian cell lines. Therefore, we cannot rule out the possibility of infections of vertebrates, including humans. Furthermore, birds, reptiles and amphibians should be explored as potential hosts. For NANV, there is certainly potential for human exposure, as the *Culex* mosquitoes were trapped in the backyard of a house in an urban area of Iquitos. Owing to co-circulation of multiple flaviviruses in and near Iquitos, including SLEV, Ilheus virus (ILHV), YFV and all four DENV serotypes (Forshey et al., 2010; Morrison et al., 2010; Turell et al., 2005), human infection with NANV might be difficult to distinguish, as flaviviruses exhibit extensive serological cross-reactivity (Kuno, 2003). Cross-reactivity with DENV, YFV and SLEV polyclonal sera was evident by IFA, and analysis of domain III of the E gene product indicated conserved amino acid motifs, including the TXHGT and PPXGXS motifs conserved across mosquito-borne flaviviruses. Because we did not amplify the entire E gene, we were unable to compare cross-reactive epitopes in domains I and II.

The prevalence of insect-specific flaviviruses in *Culex* spp. has led to the suggestion of potential intra-vector interactions among flaviviruses. CxFV infection could potentially exclude subsequent WNV infection (Bolling et al., 2012), although this hypothesis is challenged.

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**Fig. 2.** Maximum-likelihood phylogenetic trees of members of the genus *Flavivirus*, based on partial NS5 (a) and E (b) amino acid sequences. The trees are midpoint rooted, and bootstrap values >70 % are shown. Bars, number of substitutions per site. ‘Secondary loss’ no known vector viruses are italicized, while NANV is shown in bold type and underlined. Other viruses analysed were Aedes flavivirus (AeFV), Alkhurma virus (AHFV), Apoi virus (APOIV), Bagaza virus (BAGV), Banzi virus (BANV), Boubou virus (BOUV), Bussuquara virus (BSQV), cell fusing agent virus (CFAV), Cacipacore virus (CPCV), Culex flavivirus (CxFV), dengue virus type 1 (DENV-1), dengue virus type 2 (DENV-2), dengue virus type 3 (DENV-3), dengue virus type 4 (DENV-4), Edge Hill virus (EHV), Entebbe bat virus (ENTV), Gadgets Gully virus (GGYV), Ilheus virus (ILHV), Japanese encephalitis virus (JEV), Kadam virus (KADV), Kedougou virus (KEDV), Kyasanur forest disease virus (KFDV), Kokobera virus (KOKV), Kamiti River virus (KRV), Kunjin virus (KUNV), Lammi virus (LAMV), Langat virus (LGTV), louping ill virus (LIV), Montana myotis leukoencephalitis virus (MMLV), Modoc virus (MODV), Murray Valley encephalitis virus (MVEV), Nakwiro virus (NAKV), Naranjal virus (NJLV), Nounané virus (NOUV), Powassan virus (POWV), Quang Binh virus (QBV), Rio Bravo virus (RBV), Rocío virus (ROCV), Sepik virus (SEPV), St. Louis encephalitis virus (SLEV), Spondweni virus (SPOV), tick-borne encephalitis virus (TBEV), Usutu virus (USUV), West Nile virus (WNV), yellow fever virus (YFV), Yokose virus (YOKV) and Zika virus (ZIKV).
by the existence of co-infections in field-collected mosquitoes (Newman et al., 2011) and enhanced WNV replication during co-inoculation with CxFV in other systems (Kent et al., 2010). Unlike Culex (Cul.) spp., Culex (Mel.) spp. are not often incriminated as vectors of flaviviruses, except for isolations of BSQV (Srihongse & Johnson, 1971) and Naranjal virus (Calisher et al., 1983). Culex (Mel.) spp. have been shown to be vectors for other viral pathogens in Peru, including alphaviruses [e.g. Venezuelan equine encephalitis virus (VEEV)] and orthobunyaviruses (e.g. Group C and Guama Group) and were the source of 85% of virus isolations in one study (Turell et al., 2005). Thus, NANV circulation is unlikely to have an impact on heterologous flaviviruses in the region, and, while there is potential for interactions with other arboviruses, it is unclear if there would be any effect on their transmission dynamics.

In conclusion, our data demonstrate the circulation of a novel flavivirus in the Amazon basin of Peru. Along with NOUV and LAMV, NANV represents a novel phenotype among flaviviruses: phylogenetic placement with pathogenic mosquito-borne flaviviruses but with replication restriction in mammalian cells. Comparative analysis with other mosquito-borne flaviviruses could aid in identifying viral host range determinants and mechanisms of evolution and emergence of medically important flaviviruses.

**METHODS**

**Mosquito collection.** Mosquitoes were collected between March 2008 and May 2010 in CO2-baited CDC light traps placed in peri-domestic settings on three consecutive nights each month in distinct zones of Iquitos, including Nuevo Versailles, San Juan and Bellavista-Domestic settings on three consecutive nights each month in distinct

**Virus isolation and immunofluorescence assays.** Mosquito pools were homogenized for 2 min at 30 cycles s⁻¹ in 1 ml Earle’s modified Eagle’s minimal essential medium containing 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 1 mM sodium pyruvate, and 2 % (v/v) FBS in a Mixer Mill (model MM 300; Qiagen), using one 3 mm tungsten carbide bead per pool. Supernatant was inoculated onto C6/36 Aedes albopictus cells and Vero-76 African green monkey cells and incubated at 33 °C (C6/36 cells) and 37 °C (Vero-76 cells) as described by Caceda & Kochel (2007). Inoculated cells were checked daily for signs of cytopathic effects. After 10 days, the cells were harvested, reconstituted with PBS, spotted onto slides and tested for viral antigen by indirect IFA. Briefly, screening of the slides was performed using polyclonal antibodies targeted against antigens from the following arthropod-borne viruses: DENV-1, YFV, VEEV, Oropouche virus, Mayaro virus, Caraparu virus, SLEV and Eastern equine encephalitis virus. Because of the immunological cross-reaction often observed for flavivirus family members, positive IFA screening results using polyclonal flavivirus antibodies were followed by more specific tests using YFV, DENV-1, DENV-2, DENV-3 and DENV-4 monoclonal antibodies (Forsey et al., 2010). Antibodies were incubated on slides for 1 h at 37 °C and washed twice in PBS prior to incubation with appropriate FITC-labelled secondary antibodies (goat anti-mouse IgG; Sigma) for 1 h at 37 °C. After incubation with the secondary antibodies, the slides were washed as described above, and cells were examined for the presence of antigen by using a fluorescence microscope.

**Sequence amplification.** RNA was extracted from 140 μl cell culture supernatant using the QIAmp Viral RNA Extraction kit (Qiagen), following the manufacturer’s instructions. For analysis of the NS5 gene region, RT-PCR amplification was performed using primers specific for the flavivirus NS5 region: F9 (corresponding to base pairs 8993–9018 of YF17D) and CD9 (base pairs 10056–10077 of YF17D) (Kuno et al., 1998). To amplify the E gene region, degenerate primers were adapted from previously described primers UniFor (base pairs 1274–1299 of YF17D) and UniRev (base pairs 2324–2346 of YF17D) (Gaunt & Gould, 2005): forward, 5'-TGGGGNAAATGGMGTYYGGNNTYTYG-3'; reverse, 5'-CNCGCC-HAYGDACDKRAGTCCCA-3'. RT-PCRs were performed with 1.5 mM MgCl₂, 1 μM primers, 0.2 mM dNTPs, 5 U AMV reverse transcriptase and 2.5 U Tag polymerase. RT-PCR products were purified by Centri-Sep spin columns (Invitrogen) and sequenced directly using the Big Dye sequencing kit and analysed on a 3100 Avant-Genetic Analyzer (Applied Biosystems). Supernatants from mock-inoculated C6/36 cells were included as negative controls; no cDNA amplification was detected.

**Phylogenetic analysis.** Partial NS5 and E gene regions were compared with sequences obtained from other members of the genus *Flavivirus* available in GenBank (www.ncbi.nlm.nih.gov/nuccore; Table S1 available in JGV Online). Because of the high divergence among members of the genus *Flavivirus* and resultant ambiguity in alignments, multiple approaches were taken to align flavivirus sequences and to reconstruct phylogenetic trees. Both CLUSTAL W (Thompson et al., 1994) and MUSCLE (Edgar, 2004) were used to align nucleotide and amino acid sequences in MEGA5.05 (Tamura et al., 2011). Additionally, to avoid artefacts of highly divergent sequences, Gblocks (Talavera & Castresana, 2007) were used to remove regions of high amino acid alignment ambiguity. Genetic analyses were conducted using the results of both alignment algorithms as well as the truncated alignments from Gblocks.

Uncorrected nucleotide and amino acid p-distances were calculated from pairwise comparisons in MEGA5. Phylogenetic trees were reconstructed from nucleotide alignments using ML and Bayesian methods using a general time-reversible model plus gamma-distributed rates across sites plus an invariant rate parameter. Similarily, phylogenetic trees were reconstructed from aligned amino acid sequences with the Blast editor tool. Bayesian analyses were conducted in MrBayes 3.2, with sampling every 100 generations and a burn-in of 25% of samples. Markov chains were run until the standard deviation of the split frequencies was below 0.01, for a minimum of 10⁶ generations. Convergence was assessed based on effective sample sizes greater than 200. ML analyses were conducted using PhyML (http://www.atgc-montpellier.fr/phyml/; Guindon et al., 2005, 2010), with 100 bootstrap replicates to assess confidence at nodes. Additionally, NJ and maximum-parsimony (MP) methods were used for nucleotide and amino acid-based tree reconstruction, conducted in MEGA5.05. For NJ tree reconstruction, the Tamura–Nei algorithm was used. For NJ and MP approaches, confidence at nodes was assessed using the bootstrap procedure with 1000 resampling replicates.

**Host range in vitro.** African green monkey cells (Vero-76, Vero-E6), baby hamster kidney cells (BHK), non-malignant monkey kidney cells (LLCMK), Madin-Darby canine kidney cells (MDCK), human lung adenocarcinoma epithelial cells (A549) and human embryo rhabdomyosarcoma cells (RD) were inoculated with cell culture supernatant of virus-infected C6/36 cells. Inoculated cells were observed daily for signs of cytopathic effects. Cell supernatants were passed twice on fresh cells, and supernatant from the second passage was screened for
virus infection using IFA and RT-PCR using primers FU1 and cFD2 (Kuno et al., 1998).

Virus amplification in mice. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set out by the Institute of Laboratory Animal Resources (1996). The protocol was approved by the NAMRU-6 Institutional Animal Care and Use Committee (protocol NMRCD-10-02). Newborn mice were injected intracerebrally with infected C6/36 cell culture supernatant (Hasebe et al., 2002; Huhtamo et al., 2009; Philip Samuel & Tyagi, 2006). Mice were checked daily for signs of disease and killed on day 14 post-inoculation. Viral replication in brain tissues was assessed by IFA and RT-PCR.

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