**First isolation of Japanese encephalitis virus genotype IV from mosquitoes in Australia**

**Supplementary Data**

**Materials and Methods**

*JEV**molecular detection, isolation, sequencing, and phylogenetic analysis*

Specific Japanese encephalitis virus (JEV) RNA detection, virus isolation and next generation sequencing (NGS) methods have been reported previously (Pyke et al., 2020a). The primers and probe used for the detection of JEV RNA by reverse transcription, TaqMan™ real-time polymerase chain reaction (RT-rPCR) have been published elsewhere (Northill et al., 2018).

An isolate of JEV from one JEV RT-rPCR positive mosquito pool collected from the Mundubbera region in March 2022 (Supplementary Table 1) was obtained. Briefly, the mosquito pool had been homogenized, clarified by centrifugation and filtered through a 0.2 µm filter as previously described (Jansen et al., 2019) before inoculation of 50-100 µL onto confluent *Aedes albopictus* C6/36 cell monolayers (American Type Culture Collection (ATCC), CRL-1660) grown in Opti-MEM® (Gibco, Life Technologies Corporation, NY) supplemented with 0.2% bovine serum albumin (Sigma Aldrich, Australia). After 7 days incubation at 28oC, viral isolation (passage 1) was confirmed by probing with an anti-JEV monoclonal antibody, 6B4A-10 (Merck, Sigma-Aldrich, Australia) in an immunofluorescent antibody assay (IFA, main manuscript Figure 1A) (Pyke et al., 2020a, Pyke et al., 2018).

The JEV isolate (QLD\_S46716\_M2022) was further passaged a second time in C6/36 cells and once in baby hamster kidney cells (BHK-21; ATCC CCL-10) before characterization by next generation sequencing using the Nextera XT kit for cDNA library construction and paired-end (2 × 150 nucleotides) sequencing using the V2 mid-output kit on a NextSeq 500 machine (Illumina, San Diego, CA) as previously described (Pyke et al., 2020b). A total of 8,633,240 raw sequencing reads were obtained and were processed using Geneious Prime® version 2023.2.1 software (Kearse et al., 2012). Near complete genome *de novo* assembly was performed using SPAdes (Bankevich et al., 2012) version 3.15.5 within Geneious before prediction of the open reading frame (ORF). The assembled near genome sequence was subjected to blastn analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and confirmed as a JEV genome. Further scrutiny of the raw sequence reads was also performed, and 5,499,462 reads were mapped to a JEV reference genome, JEV strain JKT6468 isolated in Indonesia, 1981 (GenBank accession number AY184212.1).

Phylogenetic analysis was performed on 247 complete JEV ORFs to compare QLD\_S46716\_M2022 (GenBank accession number OR965960) with other available JEV genomic sequences. Multiple nucleotide sequence alignments were performed using the Multiple Alignment using Fast Fourier Transform (MAFFT) program version 7.490 and Geneious Prime® version 2023.2.1 software (Kearse et al., 2012). A maximum likelihood (ML) tree (main manuscript Figure 1B) was inferred from the 247 JEV complete coding regions using IQ-Tree (Nguyen et al., 2015) version 2.1.2 and the generalized time-reversible, empirical base frequencies, invariant sites and FreeRate model with 4 categories (GTR + F + I + R4) nucleotide substitution model. The tree was constructed with bootstrap support estimated from 1,000 replicates and graphically viewed in FigTree version 1.4.4 (<http://tree.bio.ed.ac.uk/software/figtree/>).

**Supplementary Table 1.** Details of trap collections from the Wide Bay region of Queensland, Australia, from which Japanese encephalitis virus (JEV) was detected by reverse transcription, TaqMan™ real-time polymerase chain reaction (RT-rPCR).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Date of collection | Number traps deployed | Number of mosquitoes in JEV RNA positive trap | Mosquito species composition of positive trapa | Number pools processed | Number pools positive |
| 15/3/2022 | 10 | 418 | *Aedes vittiger* (373), *Anopheles annulipes* (2), *An. bancroftii* (2), *Culex annulirostris* (28), *Cx. gelidus* (3), *Coquillettidia xanthogaster* (5), *Mansonia uniformis* (5) | 9 | 8b |
| 5/4/2022 | 14 | 173 | *Ae. alternans* (1), *Ae. lineatopennis* (1), *An. amictus* (3), *Cx. annulirostris* (29), *Cx. australicus* (4), *Cx. gelidus* (134), *Lutzia halifaxii* (1) | 4 | 2 |
| 184 | *Ae. alternans* (3), *Cx. annulirostris* (51), *Cx. australicus* (3), *Cx. gelidus* (127) | 4 | 4 |
| 19/4/2022 | 10 | 21 | *An. amictus* (1), *Cx. annulirostris* (1), *Cx. gelidus* (19) | 1 | 1 |

aThe numbers in parentheses refer to the number of mosquitoes of each species that was identified in the trap collection.

bJEV GIV was isolated from one JEV positive pool of mosquitoes collected from the Mundubbera region.

**References for supplementary data**

Bankevich A, Nurk S, Antipov D, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 2012;19(5):455-477; doi: 10.1089/cmb.2012.0021.

Jansen CC, Shivas MA, May FJ, et al. Epidemiologic, entomologic, and virologic factors of the 2014-15 Ross River virus outbreak, Queensland, Australia. Emerg Infect Dis 2019;25(12):2243-2252; doi: 10.3201/eid2512.181810.

Kearse M, Moir R, Wilson A, et al. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012;28(12):1647-1649; doi: 10.1093/bioinformatics/bts199.

Nguyen LT, Schmidt HA, von Haeseler A, et al. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol 2015;32(1):268-274; doi: 10.1093/molbev/msu300.

Northill JA, Finger M, Lyon M, et al. Japanese encephalitis virus real-time RT-PCR V.3 Protocolsio 2018; doi: dx.doi.org/10.17504/protocols.io.r23d8gn.

Pyke AT, Choong K, Moore F, et al. A case of Japanese encephalitis with a fatal outcome in an Australian who traveled from Bali in 2019. Trop Med Infect Dis 2020a;5(3); doi: 10.3390/tropicalmed5030133.

Pyke AT, Gunn W, Taylor C, et al. On the home front: specialised reference testing for dengue in the Australasian region. Trop Med Infect Dis 2018;3(3); doi: 10.3390/tropicalmed3030075.

Pyke AT, McMahon J, Burtonclay P, et al. Genome sequences of chikungunya virus strains from Bangladesh and Thailand. Microbiol Resour Announc 2020b;9(2); doi: 10.1128/MRA.01452-19.