Pathology (■ xxxx) **xxx(xxx)**, xxx

CORRESPONDENCE

A real-time PCR assay for Japanese encephalitis virus (JEV) genotype IV as a public health laboratory response to an emerging outbreak in Australia

To the Editor,

In early 2022 the mosquito-borne flavivirus, Japanese encephalitis virus (JEV), extended its geographical distribution for the first time into the southern temperate regions of Australia, resulting in more than 40 cases of human infections across multiple states, with a possible sentinel case identified in 2021.[1] Earliest human cases during the outbreak were identified as JEV genotype IV with a hemi-nested polymerase chain reaction (PCR) utilising primers targeting the whole *Flaviviridae* family[2] and confirmed by sequencing at the Victorian Infectious Diseases Reference Laboratory (VIDRL) Melbourne, Australia. This circulating JEV genotype (NCBI GenBank accession number ON624132) evaded detection by high-throughput real-time PCR assay due to multiple single-nucleotide polymorphisms (SNPs) in primers and probe binding sites (in-house and published).[3,4] Prior to this outbreak, genotype IV JEV had been rarely described with only five published whole genome sequences. Additional qPCR assays were thus assessed for the detection of genotype IV JEV using de novo primer design or adaptation of published assays (Table 1).[5] Primer sequences are included in Table 2.

RNA extraction was performed using QIAamp Viral RNA Mini Kit or QIAamp 96 Virus QIAcube HT Kit on the QIACube HT platform (Qiagen, Australia) as per the manufacturer's instructions. Lysis buffer included low copy bovine diarrhoeal virus (BVDV), used as an internal control as previously published.[6] For JEV genotype IV material, RNA extraction was performed using MagMax 96 RNA Isolation Kit (Thermo Fisher Scientific, Australia). Reverse transcription of RNA to complimentary DNA (cDNA) was performed using random hexamer priming with SensiFAST cDNA reaction mix (Bioline Reagents, Australia), then cycled using an Applied Biosystems SimpliAmp thermal cycler (Thermo Fisher Scientific) as follows: 25°C (10 min), 42°C (15 min) and 85°C (5 min).

Detailed methods for conventional pan-flavivirus PCR are published elsewhere[2] and primers and probes used in the various methods are listed in Table 2. Thermocycling was performed using an Applied Biosystems VeritiPro 96-Well Thermal Cycler (Thermo Fisher Scientific) with the following conditions. For first round reaction: 95°C for 3 min followed by 35 cycles of 95°C (20 s), 54°C (20 s) and 72°C (30 s), taken to 72°C (5 min) and held at 4°C. For second round reaction: 95°C for 3 min followed by 25 cycles of 95°C (20 s), 53°C (20 s) and 72°C (30 s), taken to 72°C (5 min) and held at 4°C. JEV detection was confirmed by sequencing the second-round PCR amplified product using SeqStudio Genetic Analyzer System (Thermo Fisher Scientific) and alignment of sequence using GenBank Basic Local Alignment Tool (BLAST).

For qPCR assays (primers and probes listed in Table 2), 3 μL of cDNA template and master-mix (Precision FAST qPCR Master Mix with low ROX; Primer Design, UK) containing primers (0.9 μM) and probe (0.2 μM) were added to a final reaction of volume of 20 μL for thermocycling using an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) as follows: 95°C for 2 min followed by 45 cycles of 95°C (5 s), and 60°C (24 s). All PCR assays included a no template control, virus-positive control and BVDV internal control.[2,5]

These qPCR assays were able to detect the virus from clinical samples with no cross-reactivity with other viruses or non-specific amplification from cerebrospinal fluid samples pre-dating JEV emergence in Australia.

Our experience highlights a number of diagnostic gaps in JEV outbreak response, namely: (1) a lack of JEV genomic

Table 1 Assay performance characteristics.

Assay	Detection of viral isolate		Detection in clinical samples		Cross-reactivity	
	Genotype I[a]	Genotype IV[b]	JEV genotype[c] IV positive	JEV genotype IV negative (pre-outbreak)	Flaviviruses[d]	Pathogens causing encephalitis[e]
Pan-flavivirus RT-PCR[2]	Yes	Yes	3/3 (100%)[f]	0/40 (0%)	Not tested	None
Original JEV RT-qPCR[3]	Yes	No	–	–	–	–
JEV Universal RT-qPCR[5]	Yes	Yes	3/3 (100%)	0/40 (0%)	None	None
VIDRL1 JEV GIV RT-qPCR	No	Yes	2/3 (67%)	0/40 (0%)	None	None
VIDRL2 JEV GIV RT-qPCR	Yes	Yes	3/3 (100%)	0/40 (0%)	None	None
Modified JEV GIV RT-qPCR[5]	Yes	Yes	2/3 (67%)	0/40 (0%)	None	None

[a] Derived from clinical isolate described in Huang *et al.*[4]
[b] Porcine-derived JEV genotype IV material.
[c] Cerebrospinal fluid (n=2) and brain tissue (n=1).
[d] Assessed for amplification of nucleic acid from dengue virus types 1−4, Kunjin virus, West Nile virus, Murray Valley encephalitis virus, Kokobera virus, Zika virus, or yellow fever virus.
[e] Assessed for amplification of nucleic acid from herpes simplex viruses 1 and 2, human herpes virus 6, varicella zoster virus and enterovirus (Coxsackievirus B2).
[f] Positivity via this assay was used to identify samples as JEV genotype IV for testing here.

Print ISSN 0031-3025/Online ISSN 1465-3931

Please cite this article as: Martin GE et al., A real-time PCR assay for Japanese encephalitis virus (JEV) genotype IV as a public health laboratory response to an emerging outbreak in Australia, Pathology, https://doi.org/10.1016/j.pathol.2023.02.006

Table 2 Assay primers and probes.

Assay	Assay type	Primers and probes	JEV genome target	Reference
Pan-flavivirus	Conventional	First round Forward: 5′-AACATGATGGGVAARMGDGAGAA-3′ Reverse: 5′-GTGTCCCADCCDGCKGTRTCATCNG-3′ Second round Forward: 5′-AARGGRAGYMGNGCHATHTGGT-3′ Reverse: 5′-GTGTCCCADCCDGCKGTRTCATCNG-3′	NS5	Russell (2018)[2]
Original JEV RT-qPCR	Real-time	Forward: 5′-ATCTGGTGYGGYAGTCTCA-3′ Reverse: 5′-CGCGTAGATGTTCTCAGCCC-3′ Probe: 5′-CGGAACGCGATCCAGGGCAA-3′	NS5	Pyke (2004)[3]
JEV Universal RT-qPCR	Real-time	Forward: 5′-GCCACCCAGGAGGTCCTT-3′ Reverse: 5′-CCCCAAAACCGCAGGAAT-3′ MGB probe: 5′-CAAGAGGTGGACGGCC-3′	NS1	Shao (2018)[5]
VIDRL1 JEV GIV RT-qPCR[a]	Real-time	Forward: 5′-ACGCAATACAACARCGTCTGA-3′ Reverse: 5′-AAGTTTGGTACCGCACTGGAA-3′ MGB probe: 5′-CTGGCACCGACACGA-3′	NS3	
VIDRL2 JEV GIV RT-qPCR[a]	Real-time	Forward: 5′-GGCCTTCTGGTGATGTTTC-3′ Reverse: 5′-TAGCACTACGTACCTCRCCARAT-3′ MGB probe: 5′-TGACAGTTCCTGCGGTTT-3′	NS2A	
Modified JEV GIV RT-qPCR[b]	Real-time	Forward: 5′-TTCAATATGGACGGTGCACAY-3′ Reverse: 5′-CCRTGYGTCTGGACCGACA-3′ Probe: 5′-AACCAGACACTCYAAGAGAAGYAGGAGATC-3′	M	Shao (2018)[5]

All primers/probes manufactured by Bioneer Pacific (Bioneer Corporation, Korea).
MGB, minor groove binding.
[a] Newly designed primer sets.
[b] Modified reverse primer and probe nucleotide sequence from published sequences due to SNPs in these sites in the genotype IV isolated in Australia. Sequences as published[5] were unable to detect porcine-derived JEV genotype IV material and are not presented here.

sequencing to inform circulating strains; (2) the importance of periodic re-evaluation of molecular diagnostic methods; (3) a lack of positive control material for diagnostic assay design and evaluation; and (4) difficulties in obtaining a molecular diagnosis for JEV meaning heavy reliance on serological assays with significant cross reactivity.

To ameliorate the problem of real-time PCR primer dropout due to JEV sequence diversity, the use of generic primers targeting conserved flavivirus genomic regions, as described, may serve as a good supplementary assay. This approach has the added benefit of allowing for detection of other co-circulating or emerging flaviviruses due to climatic changes. JEV viraemia in humans is often transient and of low magnitude,[7] limiting diagnosis by PCR. As human infections often represent spill over events, animal surveillance is of paramount importance in informing public health and environmental control measures. Sequencing and viral culture from amplification hosts (e.g., sentinel pigs) would provide critical material and information to human laboratory diagnostics, highlighting the importance of a one health approach.

Conflicts of interest and sources of funding: The authors state that there are no conflicts of interest to disclose.

Genevieve E. Martin[1,2], Thomas Tran[1], Georgina Papadakis[1], Paul Kinsella[1,2], Julian Druce[1], Leon Caly[1,2], Deborah A. Williamson[1,2], Chuan Kok Lim[1,2]

[1]Victorian Infectious Diseases Reference Laboratory, Royal Melbourne Hospital, and Department of Medicine, University of Melbourne, at the Peter Doherty Institute for Infection and Immunity, Melbourne, Vic, Australia; [2]Department of Infectious Diseases, University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Vic, Australia

Contact Dr Chuan Kok Lim.
E-mails: chuan.lim1@unimelb.edu.au, chuan.lim@mh.org.au

1. Waller C, Tiemensma M, Currie BJ, Williams DT, Baird RW, Krause VL. Japanese encephalitis in Australia - a sentinel case. *N Engl J Med* 2022; 387: 661−2.
2. Russell JS, Caly L, Kostecki R, *et al*. The first isolation and whole genome sequencing of Murray Valley Encephalitis virus from cerebrospinal fluid of a patient with encephalitis. *Viruses* 2018; 10: 319.
3. Pyke AT, Smith IL, van den Hurk AF, *et al*. Detection of Australasian flavivirus encephalitic viruses using rapid fluorogenic TaqMan RT-PCR assays. *J Virol Methods* 2004; 117: 161−7.
4. Huang GKL, Tio SY, Caly L, *et al*. Prolonged detection of Japanese Encephalitis virus in urine and whole blood in a returned short-term traveler. *Open Forum Infect Dis* 2017; 4: ofx203.
5. Shao N, Li F, Nie K, *et al*. TaqMan real-time RT-PCR assay for detecting and differentiating Japanese Encephalitis virus. *Biomed Environ Sci* 2018; 31: 208−14.
6. Tran T, Chien BT, Papadakis G, *et al*. Respiratory virus laboratory pandemic planning and surveillance in central Viet Nam, 2008−2010. *West Pac Surveill Response J* 2012; 3: 49−56.
7. Bharucha T, Sengvilaipaseuth O, Vongsouvath M, *et al*. Development of an improved RT-qPCR assay for detection of Japanese Encephalitis virus (JEV) RNA including a systematic review and comprehensive comparison with published methods. *PLoS One* 2018; 13: e0194412.

DOI: https://doi.org/10.1016/j.pathol.2023.02.006

Please cite this article as: Martin GE et al., A real-time PCR assay for Japanese encephalitis virus (JEV) genotype IV as a public health laboratory response to an emerging outbreak in Australia, Pathology, https://doi.org/10.1016/j.pathol.2023.02.006