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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

Tehmina Bharucha[1,2,3]*, Onanong Sengvilaipaseuth[1], Manivanh Vongsouvath[1], Malavanh Vongsouvath[1], Viengmon Davong[1], Phonepasith Panyanouvong[1], Géraldine Piorkowski[4], Jeremy A. Garson[2,5], Paul N. Newton[1,6,7], Xavier de Lamballerie[4], Audrey Dubot-Pérès[1,4,5]

1 Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (LOMWRU), Microbiology Laboratory, Mahosot Hospital, Vientiane, Lao P.D.R, **2** Division of Infection and Immunity, University College London, London, United Kingdom, **3** Departments of Infectious Diseases and Microbiology, Royal Free Hospital, London, United Kingdom, **4** UMR "Unité des Virus Emergents" (UVE: Aix-Marseille Univ–IRD 190 –Inserm 1207 –IHU Méditerranée Infection), Marseille, France, **5** National Transfusion Microbiology Laboratories, NHS Blood and Transplant, London, United Kingdom, **6** Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom, **7** London School of Hygiene and Tropical Medicine (LSHTM), London, United Kingdom

* t.bharucha@doctors.org.uk

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Abstract

Background

Japanese encephalitis virus (JEV) is a major cause of encephalitis in Asia, and the commonest cause of mosquito-borne encephalitis worldwide. Detection of JEV RNA remains challenging due to the characteristic brief and low viraemia, with 0–25% of patients positive, and the mainstay of diagnosis remains detection of anti-JEV IgM antibody.

Methods

We performed a systematic review of published RT-PCR protocols, and evaluated them *in silico* and *in vitro* alongside new primers and probes designed using a multiple genome alignment of all JEV strains >9,000nt from GenBank, downloaded from the NCBI website (November 2016). The new assays included pan-genotype and genotype specific assays targeting genotypes 1 and 3.

Results

Ten RT-qPCR assays were compared, a pre-existing in-house assay, three published assays and six newly designed assays, using serial RNA dilutions. We selected three assays, one published and two novel assays, with the lowest limit of detection (LOD) for further optimisation and validation. One of the novel assays, detecting NS2A, showed the best results, with LOD approximately 4 copies/ reaction, and no cross-reaction on testing closely

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related viruses in the JEV serocomplex, *West Nile Virus* and *St. Louis Virus*. The optimised assays were validated in consecutive patients with central nervous system infections admitted to hospitals in Laos, testing paired CSF and serum samples.

Conclusions

We succeeded in developing a JEV specific RT-qPCR assay with at least 1 $\log_{10}$ improved sensitivity as compared to existing assays. Further evaluation is required, field-testing the assay in a larger group of patients.

Introduction

Japanese encephalitis virus (JEV) represents a major cause of encephalitis in Asia, with an estimated 70,000 cases per year, and 20,000 deaths [1–5]. It is a highly neurotropic arthropod-borne virus (arbovirus), a member of the genus *Flavivirus* and family *Flaviviridae*, and closely related to *West Nile virus* (WNV) and *St. Louis encephalitis virus* (SLEV).

Five JEV genotypes have been identified, G1-5, with at least 12% nucleotide difference between them, and concomitant geographical variations. G1 and G3 have been isolated and characterised most extensively in humans [6–14]. G3 was the main genotype identified in Asia until the 1990s. However, there has been a recognised genotype displacement to G1 which is now predominant [10, 15]. G1 is the only genotype that has been isolated in Laos, but it is likely that G3 co-circulates as well [6, 16].

JEV is transmitted between birds and swine in enzootic cycles with mosquito vectors (mainly *Culex* spp.) [17]. Humans are 'dead-end' hosts, infected when they encroach upon enzootic cycles, mainly involving transmission from swine, which live close to humans and have high and prolonged viraemias. The viraemia elicited in humans is typically brief and low, and in endemic areas only a small proportion of those infected (1 in 300) experience symptoms [18–20].

There are recognised limitations in current diagnostics for JEV [21–24]. The mainstay of diagnosis remains the detection of anti-JEV IgM antibody using IgM antibody capture ELISA (JEV MAC-ELISA) from blood and/or cerebrospinal fluid (CSF) samples [25–27]. However cross-reactivity of *Flavivirus* immune-assays is well-documented, as seen with *Dengue virus* infection [28]. Moreover, recent dynamics in *Flavivirus* epidemiology, seen with the epidemic of *Zika virus*, further questions our dependence on detection of anti-JEV IgM [28–31]. Field studies suggest the sensitivity of JEV MAC-ELISA is only 50–93%, and more recently the positive predictive value has also been questioned [22, 23, 32, 33]. Additionally, the use of serology for diagnostic confirmation limits knowledge of molecular epidemiology and vaccine efficacy.

Reasons for reliance on JEV MAC-ELISA are the short period of JEV viraemia in humans and potential low sensitivity of existing JEV RT-PCR techniques. Data suggest that JEV RNA is detected in blood and/or CSF in a very small proportion (0–25%) of clinical cases [32, 34]. Studies reporting higher proportions, 25–30%, involve selected patient populations, such as patients with less than 3 days onset of fever and more severe symptoms [35, 36].

We aimed to perform a systematic review of the JEV RT-PCR assays available and to compare their performance. In addition, we developed new assays, in order to obtain the highest efficiency. This required extensive sequence analysis to design an *in-silico* optimal system followed by optimization of the experimental protocol. A set of selected assays were then assessed on patient samples.

Material and methods

Systematic literature review

MEDLINE, EMBASE and Web of Science databases were searched for MESH and keywords '*Japanese encephalitis*' and 'Polymerase Chain Reaction or $ $ $PCR' upto 31st January 2017. References and citations of selected studies were also identified using Web of Science, and hand-searched in bibliographies. Inclusion criteria involved English language studies reporting validation of RT-PCR methods for detection of JEV. Panflavivirus assays were not included unless this was part of a multiplex including a specific probe for JEV.

Bioinformatic analysis, primer and probe design

JEV sequences over 9,000 nucleotides (nt) in length, identified in GenBank, were downloaded in FASTA format from the NCBI website on 12th November 2016, and aligned using MAFFT version 7 [37]. The pre-existing in-house assay (Unité des Virus Emergents, Marseille, 2014) and published RT-qPCR assays were evaluated for best fit using the sequence alignment uploaded to MEGA 7 [38]. New primers and probes were designed in areas with maximal conservation, with a GC clamp (one or more Gs or Cs within the last 5 bases from the 3' end), Tm of probes 10˚C higher than the primers, checking for hairpins, self-dimers, heterodimers using the online IDTNA 'oligoanalyzer tool' 3.1 [39, 40]. The published and newly designed assays included TaqMan® Minor Groove Binder (MGB) probes. The sequences were also submitted to nucleotide BLAST search on NCBI web server to ensure high specificity of the sequences for JEV, and predicted products were folded using the M-fold web server to check for secondary structures affecting the performance of the RT-qPCR [41, 42].

RNA standards

RNA from three JEV isolates were used for the optimisation process. Two G1 strains from Laos, JEV/CNS1326/Laos/2013 (no titre available) and JEV/CNS769/Laos/2009 ($1.3x10^6$ RNA copies/μl; GenBank KC196115, EVA 001V-02217); and one G3 strain, UVE/JEV/UNK/TW/RP9-190 ($1.2 x10^7$ RNA copies/μl; GenBank KF907505, EVA 001V-02344) [16]. Other *Flavivirus* RNAs from the JEV complex: two WNV strains, UVE/WNV/1999/US/NY 385–99 ($10^{3.57}$ TCDI50/mL, GenBank AY842931, EVA 001v-EVA140) and UVE/WNV/UNK/CF/Ar B 3573/82 ($10^{4.07}$ TCID50/mL, GenBank DQ318020, EVA 001v-EVA91), and one SLEV strain: UVE/SLEV/UNK/US/MSI-7 ($10^{4.82}$ TCID50/mL, GenBank DQ359217, EVA 001v-EVA128). Serial 1:10 dilutions of each RNA were prepared using AVE buffer containing RNA carrier (Qiagen, UK) [43]. Working aliquots were frozen at -20˚ C to avoid repeat freeze-thawing. JEV RNA dilutions were selected to cover a wide range of concentrations from positive to negative for all assays and conditions tested. For other Flaviviruses RNA, 10^{-5} dilution was chosen because it represented a moderate Cq value of approximately 30 cycles in WNV and SLEV specific qPCR assays.

Technical development of RT-qPCR assays

Comparison of RT-qPCR conditions using the pre-existing in-house system. Different factors were evaluated following step-by-step variation using the pre-existing in-house assay with dilutions of JEV G1-1326 RNA. Factors tested included: 1) Mastermix: EXPRESS One-Step SuperScript® qRT-PCR 'Express kit' (Thermo Fisher, UK), SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase 'Superscript-III kit' (Thermo Fisher, UK) and TaqMan® Fast Virus 1-Step 'Fastvirus kit' (Thermo Fisher, UK), according to manufacturers' instructions, see standard reaction mix preparation and cycling conditions in

Table 1. Standard reaction mixes and cycling conditions.

Mastermix	Preparation	Cycling Conditions
Superscript-III kit SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase	Total Reaction volume 25 μL: 1) x2 Mastermix 12.5 μL 2) Forward and reverse primers 400nM 3) Probe 160nM 4) Enzyme 0.5 μL 5) JEV RNA 5 μL	50˚C for 15 minutes 95˚C for 2 minutes X 45 (95˚C for 15 seconds + 60˚C for 45 seconds)
Express kit EXPRESS One-Step SuperScript® qRT-PCR	Total Reaction volume 20 μL: 1) x2 Mastermix 10 μL 2) Forward and reverse primers 400nM 3) Probe 160nM 4) Enzyme 2 μL 5) JEV RNA 5 μL	50˚C for 15 minutes 95˚C for 2 minutes 45 x (95˚C for 15 seconds + 60˚C for 45 seconds)
Fastvirus kit TaqMan® Fast Virus 1-Step	Total Reaction volume 25 μL: 1) X4 Mastermix 6.25 μL 2) Forward and reverse primers 400nM 3) Probe 160nM 4) JEV RNA 5 μL	50˚C for 5 minutes 95˚C for 20 seconds 45 x (95˚C for 15 seconds + 60˚C for 60 seconds)

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Table 1; 2) Reaction volumes (25 vs 50μL); 3) Sample volumes (5–16μL), see Table 2. All RT-qPCR assays for optimisation and subsequent experiments were run using a CFX96™ qPCR detection system (Biorad laboratories). Cq $>$ 40 were reported as negative, and $\leq$40 as positive.

Selection of the best performing RT-qPCR systems. The ten different systems were compared by testing them on 7 tenfold dilutions of JEV G1-1326 RNA in duplicate, and G3 RP9-190 RNA tested once, using 'Superscript-III kit' following standard conditions, see Table 1. On the basis of these results, the three RT-qPCR systems with the lowest limit of detection (LOD), i.e. highest dilution found positive for all replicates, were selected for optimisation.

Assay optimisation. The three selected RT-qPCR assays were performed on dilutions of JEV G1-1326 RNA in duplicate using 'Superscript-III kit' and step-by-step optimisation of

Table 2. Comparison of RT-qPCR conditions using the pre-existing in-house RT-qPCR assay*.

Factor	Mastermix#	Sample volume	Reaction volume	Annealing temperature	Primer concentration	Probe concentration
I Mastermix	1. Superscript-III 2. Express 3. Fastvirus	5μl	1. 25μl 2. 20μl 3. 25μl	60˚C	400nM	160nM
II Reaction volume	Superscript-III	1. 5μl 2. 10μl	1. 25μl 2. 50μl	60˚C	400nM	160nM
III Sample volume	Superscript-III	1. 10μl 2. 16μl	50μl	60˚C	400nM	160nM
	Fastvirus	1. 5μl 2. 15μl	25μl	60˚C	400nM	160nM

* All experiments were performed with G1-1326 RNA tenfold dilutions.

#Superscript-III kit: SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase; Express kit: EXPRESS One-Step SuperScript® qRT-PCR and Fastvirus kit: TaqMan® Fast Virus 1-Step.

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Table 3. Optimisation experiments performed for the three best performing RT-qPCR systems*.

Factor	Mastermix	Sample volume	Reaction volume	Annealing temperature#	Primer concentration	Probe concentration
I Annealing temperature	Superscript-III	5μl	25μl	54, 56, 58, 60, 62 and 64˚C	400nM	200nM
II Primer concentration	Superscript-III	5μl	25μl	1. 60˚C 2. 62˚C 3. 56˚C	200, 300, 400, 500, 600 and 800 nM	200nM
III Probe concentration	Superscript-III	5μl	25μl	1. 60˚C 2. 62˚C 3. 56˚C	600nM	100, 200, 300 and 400nM

* All experiments were performed with G1-1326 tenfold dilutions.

#Annealing temperature for the Pyke, NS2A and NS3 systems were 60, 62 and 56˚C respectively.

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various conditions, involving 1) annealing temperatures 54–64˚C, 2) primer concentration (200-800nM), and 3) probe concentration (100-400nM), see Table 3.

Validation

For the three selected RT-qPCR assays, efficiency, repeatability and LOD, were assessed using optimised RT-qPCR assay protocols (based on results of 2.4.1 and 2.4.3) using JEV G1-769 or JEV G3- RP9-190 RNA dilutions (10^{-3} to 10^{-10}) in triplicate, and repeated on two different days. Cross-reaction of the assays were tested using RNA from viruses in the JEV serocomplex at 10^{-5} dilutions: two WNV strains and one SLEV strain.

Evaluation using patient samples with Central Nervous System (CNS) infections

Two groups of consecutive patients with CNS infection, admitted 2009–2015, were retrospectively tested: group 1 with acute encephalitis syndrome determined using the clinical WHO criteria [44] and admitted within 7 days of fever onset to Mahosot Hospital; and group 2 with suspected CNS infection from three other hospitals in Vientiane, the Friendship, Children and Setthathirat Hospitals. CSF was collected by lumbar puncture (LP) from consenting patients without contraindications to LP, according to the judgment of the responsible physician. Blood was collected on admission for all patients. Samples were sent to the Mahosot Hospital Microbiology Laboratory. CSF and serum, after blood centrifugation, were aliquoted and stored at -80˚C.

200 μl serum and CSF samples were extracted and eluted in 60μl on a EZ1 Advanced machine using EZ1 Virus Mini Kit v2.0 (Qiagen, UK) following manufacturer's instructions. The validated JEV RT-qPCR assays were performed as per the optimised protocols for each sample. Positive and negative (no template) controls were performed for each RT-qPCR run. An internal control (MS2 phage) was added to each patient sample. After extraction, MS2 RT-qPCR was performed to control the extraction process and to exclude inhibition as previously described [45].

Sequencing

Single positive RT-qPCR results were further investigated by sending the RT-qPCR product for next-generation sequencing using Ion S5 system (Thermo Fisher, Waltham, USA), to Unité des Virus Emergents, Faculty of Medicine, Marseille, France.

Pan-flavivirus RT-PCR

The standard diagnostic algorithm at Mahosot Hospital did not include a JEV RT-PCR assay, but it did include prospective testing using a pan-flavivirus hemi-nested SybrGreen RT-qPCR with sequencing for identification. This was performed as per a published protocol [46], with results presented alongside JEV RT-qPCR results.

Panbio ELISA

Detection of anti-JEV IgM using JEV MAC-ELISA is the World Health Organisation recommended test for diagnosing JEV infection. Japanese Encephalitis-Dengue IgM Combo ELISA kit (Catalogue number E-JED01C; Panbio ELISA; Inverness Medical Innovations, Brisbane, Australia, formerly Panbio Ltd) is a commercial JEV MAC-ELISA kit that is combined with an anti-Dengue IgM test to exclude cross-reactivity. The test was performed according to the manufacturers' instructions, reported as anti-JEV or anti-Dengue IgM Positive, Equivocal or Negative [22]. For the purposes of this analysis, anti-Dengue IgM Positive or Equivocal, or anti-JEV IgM Equivocal were interpreted as anti-JEV IgM Negative.

Ethical considerations

The study was part of an ongoing study on the causes of CNS infections in Laos. Ethical clearance was granted by the Ethical Review Committee of the Faculty of Medical Sciences, National University of Laos, and the Oxford University Tropical Ethics Research Committee, Oxford, UK. Diagnostic testing was performed on anonymised and frozen serum and CSF samples previously collected. Venepuncture and LPs were performed if written consent was given by patients or their parents/guardian.

Results

Systematic review and bioinformatics analysis

Forty-six studies evaluating JEV RT-PCR assays were identified (see S1 File, S1 Fig and Table A in S2 File), including 7 assays utilising hydrolysis probes [27, 47–52]. The first published record of JEV RT-PCR was performed as a conventional RT-PCR at the National Institute of Health, Maryland, USA, in 1991 [53]. Since then, a variety of techniques have been harnessed: conventional and nested RT-PCR; and RT-qPCR using hydrolysis probes or SYBR green. Genotype-specific assays have also been reported, specifically for G1 and G3 [52, 54].

Thirteen (28%) studies reported detection of JEV RNA in patients (see Table A in S2 File). Among these studies, the median number of published JEV cases confirmed by the assay was 12 (range 1–75). These mainly involved CSF samples, but the assays also detected JEV RNA in serum. A case report has recently demonstrated detection of JEV RNA in urine, although this was not substantiated by a larger case series of 52 JEV patients [55, 56].

The primers and probes from the five published hydrolysis probe RT-qPCR assays specifically designed for detection of JEV RNA in human cases (Table C in S2 File) were aligned with the 303 complete genome sequence (see Table B in S2 File for Accession numbers, Table D in S2 File for sequence alignment). Pyke *et al*, Yang *et al* and Shirato *et al* [27, 48, 50] systems showed the best fit with the sequence alignment *in-silico*, with published validation data including genotypes tested, and were chosen for subsequent analysis. Six new primer/probe sets were also designed, targeting conserved regions of 1) all Genotypes, 2) G1 and G3 (Table 4). It is notable that the G1 and G3 assays were designed using two separate multiple genome alignments containing only strains belonging to the corresponding genotype. However, they were not exclusively targeting a specific genotype, and for this reason the assays were tested against both G1 and G3.

Table 4. Primers and probes evaluated during the study.

Author	Target (nt)	Oligonucleotides	Sequence (5'-3')*	Tm (˚C)
Pre-existing in-house	NS3 (103)	Forward	6399-TYG-AYG-CRA-GRG-TTT-ATG-CAG	61–68
		Probe 1	Fam-AGT-GGT-TTA-AGG-ATT-TTG-CAG-C-Tamra	62
		Probe 2	Fam-AGT-GGT-TCA-ARG-ACT-TTG-CAG-C-Tamra	64–66
		Reverse	6502-CAT-RCG-ACC-GAG-CAC-CTC-TA	64–67
Pyke *et al* 2004	NS5 (62)	Forward	10230-ATC-TGG-TGY-GGY-AGT-CTC-A	61–67
		Probe	Fam-CGG-AAC-GCG-ATC-CAG-GGC-AA-Tamra	69
		Reverse	10292-CGC-GTA-GAT-GTT-CTC-AGC-CC	65
Yang *et al* 2004	3' UTR (146)	Forward	10764-GGT-GTA-AGG-ACT-AGA-GGT-TAG-AGG	64
		Probe	Fam-CCC-GTG-GAA-ACA-ACA-TCA-TGC-GGC-Tamra	70
		Reverse	10910-ATT-CCC-AGG-TGT-CAA-TAT-GCT-GTT	66
Shirato *et al* 2005	5' UTR (75)	Forward	80-AGA-ACG-GAA-GAY-AAC-CAT-GAC-TAA-A	64–66
		Probe	Fam-ACC-AGG-AGG-GCC-CGG-MGB NFQ	81
		Reverse	155-CCG-CGT-TTC-AGC-ATA-TTG-AT	62
New (this study)				
NS2A (all Genotypes)–v1	NS2A (116)	Forward	3563-AGC-TGG-GCC-TTC-TGG-T	64
		Probe	Fam-CTT-CGC-AAG-AGG-TGG-ACG-GCC-A-Tamra	70
		Reverse	3675-CCC-AAG-CAT-CAG-CAC-AAG	62
NS2A)–v2	NS2A (112)	Forward	3563-AGC-TGG-GCC-TTC-TGG-T	64
		Probe	Fam- TGG-CCG-TCC-ACC-TCT-TGC-GAA-G -Tamra	70
		Reverse	3675-CCC-AAG-CAT-CAG-CAC-AAG	62
NS5 (G 1)–v1	NS5 (315)	Forward	9925-GDG-CTG-GAT-GGA-ATG-TGA	61–63
		Probe	Fam-AGG-AGA-GTG-GAT-GAC-CAC-MGB NFQ	75
		Reverse	10240-CCA-CAC-CAG-ATG-TCC-TC	60
NS5 (G 1)–v2	NS5 (315)	Forward	9925-GDG-CTG-GAT-GGA-ATG-TGA	61–63
		Probe	Fam-AGG-AGA-GTG-GAT-GAC-YAC-MGB NFQ	72–76
		Reverse	10240-CCA-CAC-CAG-ATG-TCC-TC	60
NS3 (G 3)–v1	NS3 (141)	Forward	5726-GCA-ATG-TGC-CTC-CAA-AGA-GC	65
		Probe	Fam-TCC-TAT-GAY-ACA-GAA-TAY-CCA-AA-MGB NFQ	73–78
		Reverse	5884-GTC-GAT-GAC-CCT-GCT-CGC	66
NS3 (G 3)–v2	NS3 (158)	Forward	5726-GCA-ATG-TGY-CTC-CAA-AGA-GC	63–66
		Probe	Fam-TCC-TAT-GAY-ACA-GAA-TAY-CCA-AA-MGB NFQ	73–78
		Reverse	5884-GTC-GAT-GAC-CCT-GCT-CGC	66

*Position of the oligonucleotides is related to the Nakayama strain, Accession number EF571853.

Tm: melting temperature calculated using IDTNA tool https://www.idtdna.com/calc/analyzer setting parameters as follows: Target type RNA, Oligo Conc 0.4μM, Na Conc 50mM, Mg Conc 3mM, dNTPs Conc 0.8mM. Tms for MGB (minor groove binder) probes include an additional 15˚C.

A more extensive list of all oligonucleotides evaluated *in-silico* is included in Table C in S2 File.

NS2Av2 was designed as NS2Av1 with a modification of the probe sequence as its reverse complement.

NS5v2 was designed as NS5v1 with a modification of the probe sequence, with a degeneracy Y inserted instead of C at position 16.

NS3v2 was designed as NS3v1 with a modification of the forward primer sequence, with a degeneracy Y inserted instead of C at position 9.

NS = gene coding for non-structural protein. G = genotype

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Technical development of RT-qPCR assays

Comparison of RT-qPCR conditions using the pre-existing in-house system. Experiments were performed to establish the best mastermix kits, reaction and sample volumes using the pre-existing in-house system. All the possible combinations were not performed, but the kits were evaluated with 5 tenfold dilutions of G1-1326 RNA in duplicate or triplicate to adequately compare the limits of detection.

Mastermix. Three kits were compared, Superscript-III kit, Express kit and Fastvirus kit. The LOD for Superscript-III kit and Fastvirus kit was 10^{-6}, as compared to 10^{-5} for Express kit (Table E in S2 File). The Express kit was not therefore used in further experiments.

Reaction volumes (25μl vs 50μl). The manufacturers' instructions for both Superscript-III and Fastvirus kits advise performing RT-qPCR as 50μl reaction volumes. In an effort to conserve reagents, only Superscript-III kit was used to compare two reaction volumes, 25μl and 50μl. Increasing the reaction volume from 25μl to 50μl resulted in a lower LOD, 10^{-6} vs 10^{-7} (Table E in S2 File).

Sample volumes. The standard sample volume was 5μl. As suggested by the kit manufacturer, the sample volume may be increased by not adding water in the reaction mix. For Superscript-III kit, the sample volume may be increased to 8μl in a 25μl reaction volume, equivalent to 16μl in a 50μl reaction volume. For Fastvirus kit the sample volume may be increased to 15μl in a 25μl reaction volume, vs. 30μl in a 50μl reaction volume. The results demonstrate a lower LOD (10^{-7} vs. 10^{-6}) with a larger sample volume (Table E in S2 File).

The experiments thus demonstrated that the optimal conditions involved use of the Fastvirus kit with a 30μl sample volume and 50μl reaction volume. These optimised conditions were subsequently used for the validation experiments described in Section 3.3.

Selection of the best performing RT-qPCR systems. A total of ten RT-qPCR systems, the pre-existing in-house, three published systems, and six (two versions each of three) newly designed systems, were tested on JEV G1-1326 RNA in duplicate, and G3 RP9-190 RNA once, in tenfold serial dilutions using Superscript-III under standard conditions. RT-qPCR results are presented in Table F in S2 File. Three RT-qPCR systems with the lowest LOD were selected for further optimisation: two pan-genotype assays, the Pyke and NS2A version 1 (subsequently called NS2A); and a G3 assay, NS3 version 2 (subsequently called NS3).

Assay optimisation. The Tm, primer and probe concentrations were optimised using Superscript-III under standard reaction and sample volumes.

The performance of the three selected assays at different annealing temperatures from 52 to 64˚C was assessed by testing JEV G1-1326 RNA in triplicate in tenfold serial dilutions. A three-step protocol was used for the cycling: 1) denaturation 95˚C for 0:15, 2) annealing x˚C for 0:30, and then 3) extension 68˚C for 0:30. Adjusting the annealing temperature of the thermocycling protocol improved the LOD by one $\log_{10}$ for two assays, Cq results presented in Table G in S2 File. The optimal annealing temperature for the Pyke assay was 60˚C, NS2A assay was 62˚C and NS3 assay was 56˚C (Table G in S2 File).

The assays were tested in duplicate at various combinations of forward and reverse primers concentrations, from 200-600nM, using G1-1326 RNA at 10^{-6}, results presented in Table H in S2 File. There was minimal difference between Cq results, and a concentration of 600nM for both forward and reverse primers were selected for further experiments.

The assays were tested at different probe concentrations, from 100-400nM, using G1-1326 RNA in duplicate at 10^{-6}, results presented in Table I in S2 File. 300nM was selected for further experiments.

Note that Cq values in Tables H and Table I in S2 File are not directly comparable to each other because different PCR runs employed different threshold settings.

Validation. In view of the results detailed above, the final conditions employed Fastvirus kit with a reaction volume of 50μL, sample volume of 30μl, and primer and probe concentrations of 600nM and 300nM, respectively. The optimal annealing temperature was different for each assay: 60˚C, 62˚C and 56˚C for the Pyke, NS2A and NS3 assays, respectively. This was performed as a two-step protocol, as per the Fastvirus manufacturer's instructions.

RT-qPCR was performed with serial RNA dilutions in triplicate on 2 different days, for the Pyke and the NS2A assays using G1-769 10^{-3} to 10^{-10} or for NS3 (G3 assay) with G3-RP-190

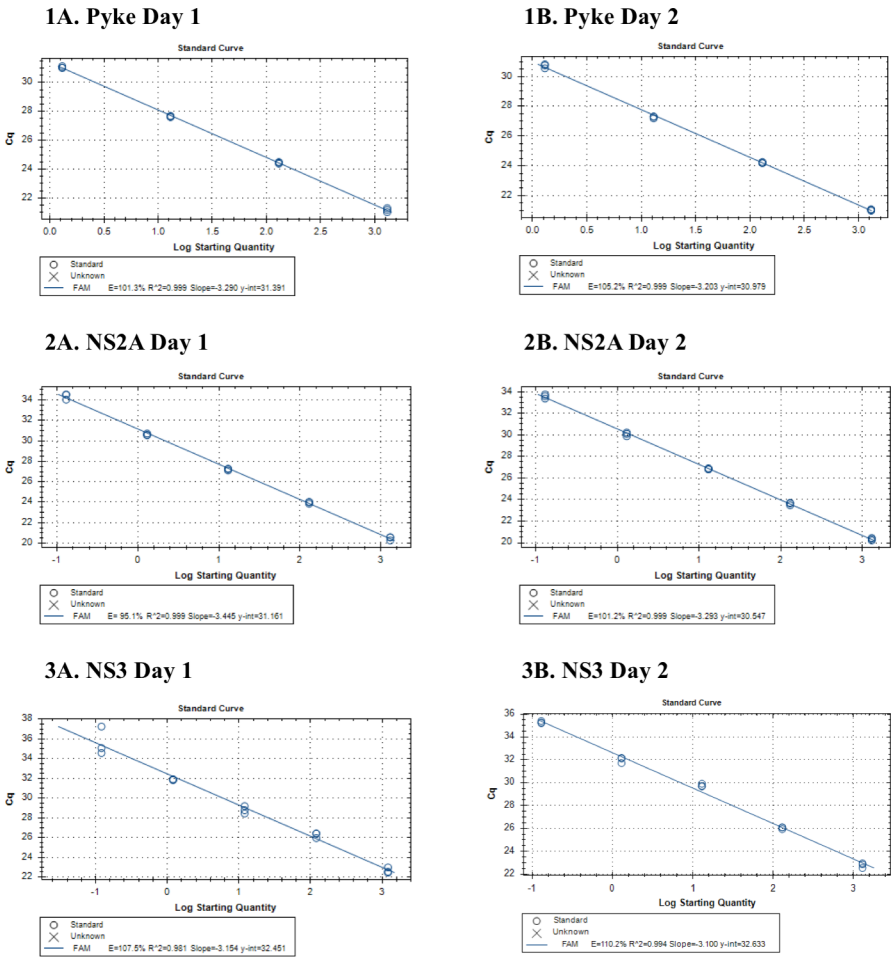

Fig 1. Standard curves of the 1) Pyke, 2) NS2A assays and 3) NS3 assays with G3-RP9-190 on (A) Day 1 and repeated on (B) Day 2. Result of the RT-qPCR run, 'Cq', is plotted against the 'log starting copies number', at the RNA dilutions detected: Pyke assay 1:10 serial dilutions of G1-769 in triplicate at 10^{-3} to 10^{-6}; NS2A assay 1:10 serial dilutions of G1-769 in triplicate at 10^{-3} to 10^{-7}; and NS3 with G3-RP-190 10^{-4} to 10^{-7}. Efficiency = $10^{-1/slope}$-1. R^2 = Correlation Coefficient. RT-qPCR performed with Fastvirus kit (TaqMan® Fast Virus 1-Step) with a reaction volume of 50μL, sample volume of 30μl, and primer and probe concentrations of 600nM and 300nM respectively. Thermocycling conditions were 50˚C for 5 minutes, 95˚C for 20 seconds and 45 x (95˚C for 15 seconds + x˚C for 60 seconds). The optimal annealing temperature 'x˚C' was different for each assay: 62˚C, 60˚C and 56˚C for the Pyke, NS2A and NS3 assays respectively.

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10^{-4} to 10^{-11}. The standard curves are shown in Fig 1 with Cq results in Table 5. The limit of detection (LOD) for the Pyke assay was 1.3 JEV RNA copies/μl or 39 copies/reaction, for the NS2A assay was $1.3x10^{-1}$ JEV RNA copies/μl or ~4 copies/reaction and for the NS3 assay was $1.2\ x10^{-1}$ JEV RNA copies/μl or ~4 copies/reaction. Similar results were obtained when testing NS2A on G3-RP9-190, see Table 5.

Efficiency was calculated from the standard curve for Day 1 and 2: 101.3% and 105.2% for the Pyke; 95.1% and 101.2% for NS2A; and 107.5% and 110.2% for NS3 (Fig 1). Assay repeatability was assessed by calculating the standard deviation (SD) for the Cq variance, presented in Table 5. SD for all assays was less than 0.40, excluding the dilution at the LOD, suggesting adequate repeatability of the assays.

No amplification was detected in testing the assays with WNV or SLEV.

Table 5. Cq results for validation of the optimised JEV RT-qPCR assays.

Assay	JEV Strain	Day	N	10^{-3}			10^{-4}			10^{-5}			10^{-6}			10^{-7}			10^{-8}			10^{-9}			10^{-10}			10^{-11}		
				Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD	Cq	Mean	SD
Pyke	G1	1	1	21.30	21.16	0.14	24.45	24.44	0.05	27.66	27.65	0.05	31.14	31.06	0.07	39.28	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-			
Pyke	G1	1	2	21.17			24.39			27.69			31.03			35.24			No Cq			No Cq			No Cq					
Pyke	G1	1	3	21.02			24.49			27.60			31.02			No Cq			No Cq			No Cq			No Cq					
Pyke	G1	2	1	21.00	21.05	0.05	24.20	24.22	0.04	27.19	27.27	0.07	30.55	30.71	0.14	34.27	35.95	2.52	No Cq	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-			
Pyke	G1	2	2	21.05			24.27			27.30			30.75			38.84			No Cq			No Cq			No Cq					
Pyke	G1	2	3	21.09			24.19			27.32			30.82			34.73			No Cq			No Cq			No Cq					
NS2A	G1	1	1	20.57	20.47	0.18	24.00	23.96	0.10	27.10	27.21	0.10	30.58	30.62	0.09	34.03	34.36	0.29	36.98	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-			
NS2A	G1	1	2	20.57			23.85			27.29			30.72			34.51			No Cq			No Cq			No Cq					
NS2A	G1	1	3	20.26			24.04			27.24			30.56			34.54			36.27			No Cq			No Cq					
NS2A	G1	2	1	20.44	20.33	0.12	23.46	23.60	0.13	26.91	26.84	0.06	30.09	30.06	0.18	33.58	33.57	0.19	39.83	37.60	1.99	No Cq	No Cq	-	No Cq	No Cq	-			
NS2A	G1	2	2	20.33			23.71			26.82			29.87			33.37			36.93			No Cq			No Cq					
NS2A	G1	2	3	20.21			23.64			26.80			30.22			33.75			36.03			No Cq			No Cq					
NS2A	G3	1	1													30.51	30.65	0.20	34.06	34.18	0.17	No Cq	No Cq	-	No Cq	No Cq	-			
NS2A	G3	1	2													30.79			34.30			39.67			No Cq					
NS3	G3	1	1				22.46	22.66	0.28	25.96	26.27	0.27	28.78	28.81	0.38	31.85	31.87	0.04	34.57	35.63	1.43	38.49	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-
NS3	G3	1	2				22.98			26.42			28.45			31.84			37.25			38.25			No Cq			No Cq		
NS3	G3	1	3				22.55			26.42			29.20			31.92			35.07			No Cq			No Cq			No Cq		
NS3	G3	2	1				22.95	22.78	0.21	26.08	26.04	0.09	29.72	29.77	0.13	31.72	32.01	0.25	35.22	35.30	0.10	39.85	No Cq	-	No Cq	No Cq	-	No Cq	No Cq	-
NS3	G3	2	2				22.85			25.94			29.68			32.18			35.27			No Cq			No Cq			No Cq		
NS3	G3	2	2				22.54			26.10			29.92			32.12			35.41			No Cq			No Cq			No Cq		

Dilution series performed using JEV G1 RNA (JEV/CNS769/Laos/2009; $1.3x10^6$ RNA copies/μl; GenBank: KC196115, EVA 001V-02217) and JEV G3 RNA (UVE/JEV/UNK/TW/RP9-190; $1.2 x10^7$ RNA copies/μl; GenBank KF907505, EVA 001V-02344) with triplicates on 2 different days. N = replicate number. LOD (highest dilution found positive by all replicates) is shaded in grey. RT-qPCR performed with Fastvirus kit (TaqMan® Fast Virus 1-Step) with a reaction volume of 50μL, sample volume of 30μl, and primer and probe concentrations of 600nM and 300nM respectively. Thermocycling conditions were 50˚C for 5 minutes, 95˚C for 20 seconds and 45 x (95˚C for 15 seconds + x˚C for 60 seconds). The optimal annealing temperature 'x˚C' was different for each assay: 62˚C, 60˚C and 56˚C for the Pyke, NS2A and NS3 assays respectively.

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Evaluation using patient samples with Central Nervous System (CNS) infections

123 patients were tested with the three optimised RT-qPCR assays (conditions as described in Section 3.2.1), 58 patients from group 1 (2011–2014) and 65 from group 2 (2008–2015), including a total of 95 serum and 120 CSF samples. 35.8% (44/123) of the patients were found anti-JEV IgM positive by MAC ELISA. Positive RT-qPCR results are presented in Table 6, alongside results for JEV MAC-ELISA, hemi-nested panflavivirus RT-qPCR and RT-qPCR sequencing results. None of the samples were considered inhibitory according to the results of MS2 testing. Two patients (2 CSF samples) were positive using the NS2A assay, of which 1 (1 CSF) was also positive by the NS3 assay. These 2 patients were known positives by cell inoculation and/or hemi-nested panflavivirus assay. 12 (8 CSF and 4 serum samples) were positive by the Pyke assay alone. As 11/12 (92%) of these patients were not previously detected by the panflavivirus assay or cell inoculation, the RT-qPCR products were sent for sequencing. This revealed that the amplicons generated by the Pyke assay with these clinical samples consisted of primer multimers and primer/probe concatemers resulting from non-specific amplification, possibly related to the unusually high GC content at 3' end of the reverse primer, GCCC.

Discussion

This systematic review confirmed that the detection of JEV RNA in suspected human cases by RT-PCR is uncommon. Forty-six studies were identified, involving a variety of RT-PCR techniques, however only a quarter had any published record of detection of JEV RNA in human samples. It was common for an article title to poorly identify the study as evaluating a JEV RT-PCR method, and while every effort was made to hand-search publications, it is possible that the search was not 100% comprehensive. Additionally, studies utilising the assays may not have cited them.

Articles rarely adhered to the MIQE guidelines for evaluation of RT-PCR methods, such as reporting accession numbers of JEV strains used or details of the optimisation process [39]. Many were only optimised and validated on G3, whereas G1 is now the predominant strain. It is also important to differentiate studies developing RT-qPCR for detection in mosquitos as opposed to humans, as they are not consistently interchangeable. Mosquito surveillance may require a cost-effective and rapid method, but in clinical diagnostics analytical accuracy is of utmost important. To this end, a number of studies detecting JEV RNA in clinical samples incorporated nested RT-PCR methods. Although nested RT-PCR may improve both analytical sensitivity and specificity, it is cumbersome and prone to cross-contamination. In the study by Meiyu *et al* [57], it was reported that out of 52 clinical JEV cases, an extraordinary 45 (87%) were PCR positive for JEV RNA. However, there was no additional evidence to support the diagnosis, such as repeat testing, sequencing of the isolates or alternative confirmation. Existing RT-qPCR assays detect JEV RNA in 0–25% of clinical cases.

It is for these reasons that we focussed on identifying and validating the most effective RT-qPCR assay. Hydrolysis probe assays are the most extensively used methods for nucleic acid detection, both for clinical and research diagnostic purposes. From the results of the systematic review, 3 published systems incorporating hydrolysis probes were selected on the basis of best matching *in silico*. Assessment with JEV RNA dilutions of G1 and G3 showed the lowest LOD was obtained with the Pyke system: 2 logfold below both the Yang and Shirato assays with G1, and 1 logfold below both with G3 [27, 50]. It is recognised that the conditions employed were not identical to those published, and it would have been interesting to compare the validated assays with existing assays performed with published conditions. Validation experiments confirmed good RT-qPCR efficiency and LOD of 1.3 JEV RNA copies/μl or 39 copies/reaction. However, in a study of patient samples with paired serum and CSF, all the positive RT-qPCR

Table 6. Evaluation of the three optimised RT-qPCR assays using patient samples with Central Nervous System (CNS) infections. Patients with RT-qPCR 'positive' test results.

Number	Age	Sex	Serology Results (PU)*			JEV RT-qPCR Results (Cq)**						Pan-flavivirus nested RT-qPCR		Sequencing Results
			Admission Serum JEV IgM	Convalescent Serum JEV IgM	CSF JEV IgM	Serum			CSF					
						Pyke	NS2A	NS3	Pyke	NS2A	NS3	CSF	Serum	
1	17	F	Positive (15.3)	Positive (56.5)	Positive (32.8)	ND	ND	ND	Negative	Positive (36)	Positive (37)	Negative	Negative	JEV sequence identified◆
2	42	F	Negative (8.6)	ND	Negative (2.9)	Positive (31)	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Non-specific amplification
3	60	M	Negative (3.9)	ND	Negative (3.5)	Negative	Negative	Negative	Positive (31)	Negative	Negative	Negative	Negative	Non-specific amplification
4	60	M	Negative (3.6)	ND	Negative (3.6)	Positive (40)	Negative	Negative	Negative	Negative	Negative	ND	ND	Non-specific amplification
5	58	F	Positive (15.9)	ND	Negative (3.4)	Negative	Negative	Negative	Positive (34)	Negative	Negative	ND	ND	Non-specific amplification
6	-	-	Positive (54.6)	ND	Positive (80.8)	Negative	Negative	Negative	Positive (40)	Negative	Negative	Negative	Negative	Non-specific amplification
7	-	M	Negative (2.5)	ND	Negative (2.6)	Negative	Negative	Negative	Positive (32)	Negative	ND	ND	ND	Non-specific amplification
8	12	M	Negative (6.8)	ND	Positive (57.2)	Negative	Negative	Negative	Positive (39)	Positive (35)	Negative	Positive	ND	JEV sequence identified◆
9	26	M	Negative (5.8)	ND	Negative (3.3)	Negative	Negative	-	Positive (36)	Negative	Negative	ND	ND	Non-specific amplification
10	27	M	Negative (4.6)	ND	Negative (3.6)	Positive (28)	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Non-specific amplification
11	33	M	Negative (3.2)	ND	Negative (2.8)	Positive (40)	Negative	Negative	Negative	Negative	Negative	ND	ND	Non-specific amplification
12			Negative (6.4)	ND	Negative (4.9)	Negative	Negative	Negative	Positive (36)	Negative	Negative	ND	ND	Non-specific amplification
13	-	-	Positive (27.8)	ND	Positive (29.9)	Negative	Negative	Negative	Positive (32)	Negative	Negative	Negative	Negative	Non-specific amplification

* JEV-Dengue IgM Combo ELISA kit (Panbio ELISA), Inverness Medical Innovations, Brisbane, Australia (formerly Panbio Ltd.). PU = Panbio Unit, calculated from OD according to manufacturer's instruction. JEV IgM Positive, Negative or Equivocal cut-offs calculated according to the manufacturer's instructions.

** Pyke, NS2A and NS3 JEV RT-qPCR assays were reported as negative if the Cq>40 or not detected.

◆ JEV sequence identified as part of a previous study, for Patient 1 by sequencing following cell culture, and for Patient 2 by sequencing of the hemi-nested qPCR product.

ND Not done.

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products that were sequenced, even results with Cq of approximately 30 showed non-specific amplification. Eleven positive samples were detected by the Pyke assay alone, of which all were sequenced and showed non-specific amplification.

The results highlight the importance of validating a newly designed RT-qPCR method against a panel of negative patients samples in addition to the no-template controls recommended by the MIQE guidelines [39]. The oligonucleotides tested in these experiments had been checked *in-silico* for specificity, and also experimentally tested for cross-reactivity against strains of the closely related viruses, WNV and SLE. All the experiments had been performed with at least one no template control per RT-qPCR plate. Nonetheless, the results of validation against patient CSF and serum samples suggested poor specificity of the Pyke assay. It is possible that further optimisation, increasing the annealing temperature and reducing the primer concentrations may have improved the specificity, although this may adversely affect sensitivity of the assay.

The new assays aimed to improve the sensitivity of detection, through targeting various conserved regions from a multiple genome alignment. The NS2A region was chosen for the pan-genotype assay, and NS5 and NS3 for the G1 and G3 systems, respectively. Two versions of each system were designed. An initial assessment showed poor performance of the NS5 assay, and this was not used in further experiments. Initial optimisation experiments demonstrated best results with Fastvirus mix, with the maximal reaction and sample volumes (although it is recognised that use of 30 μL of extract for a single assay may not be consistently possible in practice). The assays were individually optimised for annealing temperature, primer and probe concentrations. Validation showed one logfold lower LOD for the NS2A compared to the published Pyke assay for G1, NS2A assay was $1.3x10^{-1}$ JEV RNA copies/μl or ~4 copies/reaction. NS2A and NS3 showed equally good results for G3, with LOD $1.2\ x10^{-1}$ JEV RNA copies/μl or ~4 copies/reaction. Individual assays targeting highly conserved regions of G1 and G3 did not improve the sensitivity of diagnosis. Nonetheless, it may be useful to target multiple regions of the viral genome to overcome false negatives due to mutations in any single region.

A study of patient samples, including paired serum and CSF, demonstrated marginally improved detection of JEV RNA as compared to the existing hemi-nested panflavivirus RT-qPCR assay. One case was detected with the hemi-nested RT-qPCR assay, and both this and an additional case were detected by the new assays. The low number of positives is consistent with previous data. It must also be taken into account that this involved retrospective testing using samples stored for many years. The potential greater sensitivity of the NS2A as compared to the NS3 is likely due to both JEV strains detected established previously by sequencing as G1.

In conclusion, the novel pan-genotype NS2A assay and G3 NS3 assay described here showed higher analytical sensitivity than previously described RT-qPCR assays for JEV RNA. However, it will be essential to field-test them in serum, CSF and other body fluids, to evaluate their sensitivity and potential for improved diagnostic capacity in a large prospective study. Since our systematic review, there have been no further JEV specific diagnostic assays published, but there has been evidence of the potential for improved detection of JEV RNA in other body fluids, whole blood [56], throat swabs (Bharucha *et al*, submitted) and urine samples [56, 58], and this needs further evaluation.

Supporting information

S1 Fig. PRISMA flow diagram.
(DOC)

S1 File. PRISMA checklist.
(DOC)

S2 File. Table A: Overview of JEV-specific RT-PCR assays* and Sample Types Used to Detect Human JEV cases. Table B: Accession numbers of all included sequences. Table C: Complete list of Primers and Probes Evaluated *In-Silico*. Table D: Sequence alignment of Primers and Probes. Table E: Cq Results for Comparison of RT-qPCR conditions using the Pre-existing In-house JEV RT-qPCR assay. Table F: Cq Results for the Selection of the Best Performing RT-qPCR systems Using Superscript-III kit with Standard Conditions. Table G: Cq Results for Annealing Temperature Optimisation using the Pre-existing In-house JEV RT-qPCR assay. Table H: Cq Results for Primer Concentration Optimisation Experiments Using Superscript-III kit with Standard Conditions. Table I: Cq Results for Probe Concentrations Optimisation Experiments Using Superscript-III kit with Standard Conditions.
(ZIP)

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Author Contributions

Conceptualization: Tehmina Bharucha, Jeremy A. Garson, Paul N. Newton, Audrey Dubot-Pérès.

Data curation: Tehmina Bharucha.

Formal analysis: Tehmina Bharucha, Jeremy A. Garson.

Funding acquisition: Audrey Dubot-Pérès.

Investigation: Tehmina Bharucha, Onanong Sengvilaipaseuth, Malavanh Vongsouvath, Phonepasith Panyanouvong, Géraldine Piorkowski, Jeremy A. Garson, Audrey Dubot-Pérès.

Methodology: Tehmina Bharucha, Jeremy A. Garson, Xavier de Lamballerie, Audrey Dubot-Pérès.

Project administration: Tehmina Bharucha, Onanong Sengvilaipaseuth, Manivanh Vongsouvath, Viengmon Davong, Phonepasith Panyanouvong, Paul N. Newton, Audrey Dubot-Pérès.

Resources: Tehmina Bharucha, Onanong Sengvilaipaseuth, Malavanh Vongsouvath, Viengmon Davong, Paul N. Newton, Audrey Dubot-Pérès.

Software: Tehmina Bharucha, Jeremy A. Garson, Audrey Dubot-Pérès.

Supervision: Jeremy A. Garson, Paul N. Newton, Xavier de Lamballerie, Audrey Dubot-Pérès.

Writing – original draft: Tehmina Bharucha.

Writing – review & editing: Tehmina Bharucha, Onanong Sengvilaipaseuth, Manivanh Vongsouvath, Malavanh Vongsouvath, Viengmon Davong, Phonepasith Panyanouvong,

Géraldine Piorkowski, Jeremy A. Garson, Paul N. Newton, Xavier de Lamballerie, Audrey Dubot-Pérès.

References

1. Granerod J, Crowcroft NJ. The epidemiology of acute encephalitis. Neuropsychol Rehabilitation. 2007; 17:406–28.
2. Granerod J, Tam CC, Crowcroft NS, Davies NWS, Borchert M, Thomas SL. Challenge of the unknown: A systematic review of acute encephalitis in non-outbreak situations. Neurology. 2010; 75(10):924–32. https://doi.org/10.1212/WNL.0b013e3181f11d65 PMID: 20820004
3. Boucher A, Herrmann JL, Morand P, Buzele R, Crabol Y, Stahl JP, et al. Epidemiology of infectious encephalitis causes in 2016. Med Mal Infect. 2017.
4. Wang H, Liang G. Epidemiology of Japanese encephalitis: past, present, and future prospects. Therapeutics and Clinical Risk Management. 2015; 11:435–48. https://doi.org/10.2147/TCRM.S51168 PMID: 25848290
5. Campbell G, Hills S, Fischer M, Jacobson J, Hoke C, Hombach J, et al. Estimated global incidence of Japanese encephalitis:. Bulletin of the World Health Organization. 2011; 89:766–74. https://doi.org/10.2471/BLT.10.085233 PMID: 22084515
6. Saito M, Soukaloun D, Phongsavath K, Phommasack B, Makino Y. Epidemiological study of Japanese encephalitis virus in Vientiane, Lao PDR, in 1990s. TheScientificWorldJournal. 2015; 2015:235934. https://doi.org/10.1155/2015/235934 PMID: 25695095
7. Do LP, Bui TM, Hasebe F, Morita K, Phan NT. Molecular epidemiology of Japanese encephalitis in northern Vietnam, 1964–2011: genotype replacement. Virology journal. 2015; 12:51. https://doi.org/10.1186/s12985-015-0278-4 PMID: 25889499
8. Cao Y-X, Fu S-H, Wang H-Y, Pan X-L, Zhang J-B, Liang G-D. [Molecular characterization of full-length genome of Japanese encephalitis virus isolated in Liaoning Province in 2008]. Zhonghua shi yan he lin chuang bing du xue za zhi = Zhonghua shiyan he linchuang bingduxue zazhi = Chinese journal of experimental and clinical virology. 2009; 23(4):248–50. PMID: 20108764
9. Chen SP. Molecular phylogenetic and evolutionary analysis of Japanese encephalitis virus in China. Epidemiology and infection. 2012; 140(9):1637–43. https://doi.org/10.1017/S095026881100255X PMID: 22129541
10. Cherian SS, Walimbe AM. Phylogeographic analysis of Japanese encephalitis virus in India (1956–2012). Archives of virology. 2015; 160(12):3097–104. https://doi.org/10.1007/s00705-015-2599-4 PMID: 26362532
11. Gao X, Liu H, Li M, Fu S, Liang G. Insights into the evolutionary history of Japanese encephalitis virus (JEV) based on whole-genome sequences comprising the five genotypes. Virology journal. 2015; 12:43. https://doi.org/10.1186/s12985-015-0270-z PMID: 25884184
12. Gao X, Liu H, Wang H, Fu S, Guo Z, Liang G. Southernmost Asia is the source of Japanese encephalitis virus (genotype 1) diversity from which the viruses disperse and evolve throughout Asia. PLoS neglected tropical diseases. 2013; 7(9):e2459. https://doi.org/10.1371/journal.pntd.0002459 PMID: 24069502
13. Han N, Adams J, Fang W, Liu S-Q, Rayner S. Investigation of the genotype III to genotype I shift in Japanese encephalitis virus and the impact on human cases. Virologica Sinica. 2015; 30(4):277–89. https://doi.org/10.1007/s12250-015-3621-4 PMID: 26311492
14. Sarkar A, Taraphdar D, Mukhopadhyay SK, Chakrabarti S, Chatterjee S. Molecular evidence for the occurrence of Japanese encephalitis virus genotype I and III infection associated with acute encephalitis in patients of West Bengal, India, 2010. Virology journal. 2012; 9:271. https://doi.org/10.1186/1743-422X-9-271 PMID: 23153306
15. Narong N, Audrey D-P, Meriadeg Ar G, Marc S, Philippe B, Sutee Y, et al. Change in Japanese Encephalitis Virus Distribution,Thailand. Emerging Infectious Disease journal. 2008; 14(11):1762.
16. Aubry F, Vongsouvath M, Nougairede A, Phetsouvanh R, Sibounheuang B, Charrel R, et al. Complete Genome of a Genotype I Japanese Encephalitis Virus Isolated from a Patient with Encephalitis in Vientiane, Lao PDR. Genome Announc. 2013; 1(1).
17. Solomon T PA, Whitley R. Arthropod-Borne Viral Encephalitides. In: Marra C WR, Scheld M., editor. Infections of the Central Nervous System. 4th ed. Philadelphia: Lippincott Williams and Wilkins; 2014. p. 210–38.
18. Vaughn DW, Hoke CH. The epidemiology of Japanese encephalitis:prospects for prevention. Epidemiolog Rev. 1992; 14:197–221.

19. Burke DS, Chatiyanonda K, Anandrik S, Nakornsri S, Nisalak a, Hoke CH. Improved surveillance of Japanese encephalitis by detection of virus-specific IgM in desiccated blood specimens. Bulletin of the World Health Organization. 1985; 63:1037–42. PMID: 3011302

20. Burke DS, Nisalak A, Ussery MA, Laorakpongse T, Chantavibul S. Kinetics of IgM and IgG Responses to Japanese Encephalitis Virus in Human Serum and Cerebrospinal Fluid. J Infect Dis. 1985; 151 1093–9. PMID: 2987367

21. Bharucha T, Chanthongthip A, Phuangpanom S, Phonemixay O, Sengvilaipaseuth O, Vongsouvath M, et al. Pre-cut Filter Paper for Detecting Anti-Japanese Encephalitis Virus IgM from Dried Cerebrospinal Fluid Spots. PLoS neglected tropical diseases. 2016; 10(3):e0004516. https://doi.org/10.1371/journal.pntd.0004516 PMID: 26986061

22. Moore CE, Blacksell SD, Taojaikong T, Jarman RG, Gibbons RV, Lee SJ, et al. A Prospective Assessment of the Accuracy of Commercial IgM ELISAs in Diagnosis of Japanese Encephalitis Virus Infections in Patients with Suspected Central Nervous System Infections in Laos. American Journal of Tropical Medicine and Hygiene. 2012; 87:171–8. https://doi.org/10.4269/ajtmh.2012.11-0729 PMID: 22764310

23. Robinson JS, Featherstone D, Vasanthapuram R, Biggerstaff BJ, Desai A, Ramamurty N, et al. Evaluation of three commercially available Japanese encephalitis virus IgM enzyme-linked immunosorbent assays. The American journal of tropical medicine and hygiene. 2010; 83(5):1146–55. https://doi.org/10.4269/ajtmh.2010.10-0212 PMID: 21036854

24. Kakkar M, Dhole TN, Rogawski ET, Chaturvedi S. Public Health Laboratory Surveillance and Diagnosis of Japanese Encephalitis: Time to Revisit. Indian Pediatr. 2016; 53(1):33–5. PMID: 26840668

25. Organization WH, editor Manual for the Laboratory Diagnosis of Japanese Encephalitis Virus Infection2007.

26. Swami R, Ratho RK, Mishra B, Singh MP. Usefulness of RT-PCR for the diagnosis of Japanese encephalitis in clinical samples. Scandinavian journal of infectious diseases. 2008; 40:815–20. https://doi.org/10.1080/00365540802227102 PMID: 18618334

27. Yang DK, Kweon CH, Kim BH, Lim SI, Kim SH, Kwon JH, et al. TaqMan reverse transcription polymerase chain reaction for the detection of Japanese encephalitis virus. Journal of veterinary science. 2004; 5(4):345–51. PMID: 15613819

28. Johnson BW, Goodman CH, Jee Y, Featherstone DA. Differential Diagnosis of Japanese Encephalitis Virus Infections with the Inbios JE Detect and DEN Detect MAC-ELISA Kits. The American journal of tropical medicine and hygiene. 2016; 94(4):820–8. https://doi.org/10.4269/ajtmh.15-0631 PMID: 26856911

29. Kuno G. Serodiagnosis of flaviviral infections and vaccinations in humans. Adv Virus Res. 2003; 61:3–65. PMID: 14714429

30. Mishra B, Behera B. The mysterious Zika virus: Adding to the tropical flavivirus mayhem. J Postgrad Med. 2016; 62(4):249–54. https://doi.org/10.4103/0022-3859.191006 PMID: 27763483

31. Dick GW, Kitchen SF, Haddow AJ. Zika virus. I. Isolations and serological specificity. Trans R Soc Trop Med Hyg. 1952; 46(5):509–20. PMID: 12995440

32. Dubot-Peres A, Sengvilaipaseuth O, Chanthongthip A, Newton PN, de Lamballerie X. How many patients with anti-JEV IgM in cerebrospinal fluid really have Japanese encephalitis? The Lancet Infectious diseases. 2015; 15(12):1376–7. https://doi.org/10.1016/S1473-3099(15)00405-3 PMID: 26607119

33. Khalakdina A, Shrestha SK, Malla S, Hills S, Thaisomboonsuk B, Shrestha B, et al. Field evaluation of commercial Immunoglobulin M antibody capture ELISA diagnostic tests for the detection of Japanese encephalitis virus infection among encephalitis patients in Nepal. International Journal of Infectious Diseases. 2010;14.

34. Touch S, Hills S, Sokhal B, Samnang C, Sovann L, Khieu V, et al. Epidemiology and burden of disease from Japanese encephalitis in Cambodia: results from two years of sentinel surveillance. Tropical medicine & international health: TM & IH. 2009; 14:1365–73.

35. Swami R, Ratho RK, Mishra B, Singh M. Usefulness of RT-PCR for the diagnosis of Japanese encephalitis in clinical samples. Scandinavian Journal of Infectious Diseases. 2008; 40(10):815–20. https://doi.org/10.1080/00365540802227102 PMID: 18618334

36. Santhosh SR, Parida MM, Dash PK, Pateriya A, Pattnaik B, Pradhan HK, et al. Development and evaluation of SYBR Green I-based one-step real-time RT-PCR assay for detection and quantitation of Japanese encephalitis virus. Journal of Virological Methods. 2007; 143(1):73–80. https://doi.org/10.1016/j.jviromet.2007.02.011 PMID: 17403544

37. Katoh K, Misawa K, Kuma K-i, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research. 2002; 30(14):3059–66. PMID: 12136088

38. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol. 2016; 33(7):1870–4. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

39. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55 (4):611–22. https://doi.org/10.1373/clinchem.2008.112797 PMID: 19246619

40. Owczarzy R, Tataurov AV, Wu Y, Manthey JA, McQuisten KA, Almabrazi HG, et al. IDT SciTools: a suite for analysis and design of nucleic acid oligomers. Nucleic Acids Research. 2008; 36(Web Server issue):W163–W9. https://doi.org/10.1093/nar/gkn198 PMID: 18440976

41. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215(3):403–10. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

42. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003; 31(13):3406–15. PMID: 12824337

43. Read SJ. Recovery efficiencies of nucleic acid extraction kits as measured by quantitative LightCycler (TM) PCR. Molecular Pathology. 2001; 54(2):86–90. PMID: 11322169

44. World Health Organization S-EAatWP, editor World Health Organisation. Japanese encephalitis surveillance standards. In: WHO-recommended standards for surveillance of selected vaccine-preventable diseases WHO/V&B/03.012006.

45. Ninove L, Nougairede A, Gazin C, Thirion L, Delogu I, Zandotti C, et al. RNA and DNA Bacteriophages as Molecular Diagnosis Controls in Clinical Virology: A Comprehensive Study of More than 45,000 Routine PCR Tests. PloS one. 2011; 6(2):e16142. https://doi.org/10.1371/journal.pone.0016142 PMID: 21347398

46. Moureau G, Temmam S, Gonzalez JP, Charrel RN, Grard G, de Lamballerie X. A real-time RT-PCR method for the universal detection and identification of flaviviruses. Vector borne zoonotic dis. 2007; 7 (4):467–77. https://doi.org/10.1089/vbz.2007.0206 PMID: 18020965

47. Barros SC, Ramos F, Ze-Ze L, Alves MJ, Fagulha T, Duarte M, et al. Simultaneous detection of West Nile and Japanese encephalitis virus RNA by duplex TaqMan RT-PCR. Journal of Virological Methods. 2013; 193(2):554–7. https://doi.org/10.1016/j.jviromet.2013.07.025 PMID: 23892127

48. Pyke AT, Smith IL, van den Hurk AF, Northill JA, Chuan TF, Westacott AJ, et al. Detection of Australasian Flavivirus encephalitic viruses using rapid fluorogenic TaqMan RT-PCR assays. J Virol Methods. 2004; 117(2):161–7. https://doi.org/10.1016/j.jviromet.2004.01.007 PMID: 15041213

49. Huang JL, Lin HT, Wang YM, Weng MH, Ji DD, Kuo MD, et al. Sensitive and specific detection of strains of Japanese encephalitis virus using a one-step TaqMan RT-PCR technique. Journal of Medical Virology. 2004; 74(4):589–96. https://doi.org/10.1002/jmv.20218 PMID: 15484282

50. Shirato K, Miyoshi H, Kariwa H, Takashima I. Detection of West Nile virus and Japanese encephalitis virus using real-time PCR with a probe common to both viruses. J Virol Methods. 2005; 126(1–2):119–25. https://doi.org/10.1016/j.jviromet.2005.02.001 PMID: 15847927

51. van den Hurk AF, Hall-Mendelin S, Townsend M, Kurucz N, Edwards J, Ehlers G, et al. Applications of a sugar-based surveillance system to track arboviruses in wild mosquito populations. Vector borne zoonotic dis. 2014; 14(1):66–73. https://doi.org/10.1089/vbz.2013.1373 PMID: 24359415

52. Chen YY, Lin JW, Fan YC, Chiou SS. Detection and differentiation of genotype I and III Japanese encephalitis virus in mosquitoes by multiplex reverse transcriptase-polymerase chain reaction. Transboundary and emerging diseases. 2014; 61(1):37–43. https://doi.org/10.1111/j.1865-1682.2012.01369.x PMID: 23157736

53. Eldadah ZA, Asher DM, Godec MS, Pomeroy KL, Goldfarb LG, Feinstone SM, et al. Detection of flaviviruses by reverse-transcriptase polymerase chain reaction. Journal of Medical Virology. 1991; 33 (4):260–7. PMID: 1713265

54. Yang DK, Kim HH, Jo HY, Choi SS, Cho IS. Establishment of a multiplex RT-PCR for the sensitive and differential detection of Japanese encephalitis virus genotype 1 and 3. Journal of Bacteriology and Virology. 2016; 46(4):231–8.

55. Zhao H, Wang YG, Deng YQ, Song KY, Li XF, Wang HJ, et al. Japanese encephalitis virus RNA not detected in urine. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America. 2013; 57(1):157–8.

56. Mai NTH, Phu NH, Nhu LNT, Hong NTT, Hanh NHH, Nguyet LA, et al. Central Nervous System Infection Diagnosis by Next-Generation Sequencing: A Glimpse Into the Future? Open Forum Infect Dis. 2017; 4(2):ofx046. https://doi.org/10.1093/ofid/ofx046 PMID: 28480297

57. Meiyu F, Huosheng C, Cuihua C, Xiaodong T, Lianhua J, Yifei P, et al. Detection of flaviviruses by reverse transcriptase-polymerase chain reaction with the universal primer set. Microbiology and Immunology. 1997; 41(3):209–13. PMID: 9130232

58. Huang GKL, Tio SY, Caly L, Nicholson S, Thevarajan I, Papadakis G, et al. Prolonged Detection of Japanese Encephalitis Virus in Urine and Whole Blood in a Returned Short-term Traveler. Open Forum Infectious Diseases. 2017; 4(4):ofx203. https://doi.org/10.1093/ofid/ofx203 PMID: 29226169