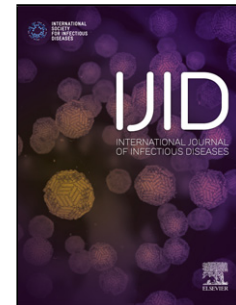


Journal Pre-proof

A need to raise the bar - A systematic review of temporal trends in diagnostics for Japanese encephalitis virus infection, and perspectives for future research

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Title: A need to raise the bar - A systematic review of temporal trends in diagnostics for Japanese encephalitis virus infection, and perspectives for future research.

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Highlights

- *Japanese encephalitis virus* (JEV) remains a leading cause of neurological infection in Asia
- A systematic review identified 20,212 published human cases of laboratory confirmed JEV infections from 205 studies.
- 15,167 (75%) of cases were confirmed with the lowest confidence diagnostic test, i.e. level 3 or 4, or level 4.
- Only 109 (53%) of the studies reported contemporaneous testing for dengue-specific antibodies.
- A fundamental pre-requisite for the control of JE is lacking --- that of a simple and specific diagnostic procedure that can be adapted for point-of-care tests and readily used throughout JE endemic regions of the world.

Abstract

Objective: Japanese encephalitis virus infection (JEV) remains a leading cause of neurological infection in Asia, largely involving individuals living in remote areas with limited access to treatment centres and diagnostic facilities. Laboratory confirmation is fundamental for the justification and implementation of vaccination programmes. We sought to review the literature on historical developments and current diagnostic capability worldwide, to identify knowledge gaps and instil urgency to address them.

Methods: Searches were performed in Web of Science and PubMed using the text word term 'Japanese encephalitis' up to 13th October 2019. Studies reporting laboratory-confirmed symptomatic JE cases in humans were included, and data on details of diagnostic tests were extracted. A JE case was classified according to confirmatory levels (1-4), where level 1 represented the highest level of confidence.

Findings: 20,212 published JE cases were identified from 205 studies. 15,167 (75%) of these positive cases were confirmed with the lowest confidence diagnostic test (level 3 or 4, or level 4). Only 109 (53%) of the studies reported contemporaneous testing for dengue-specific antibodies.

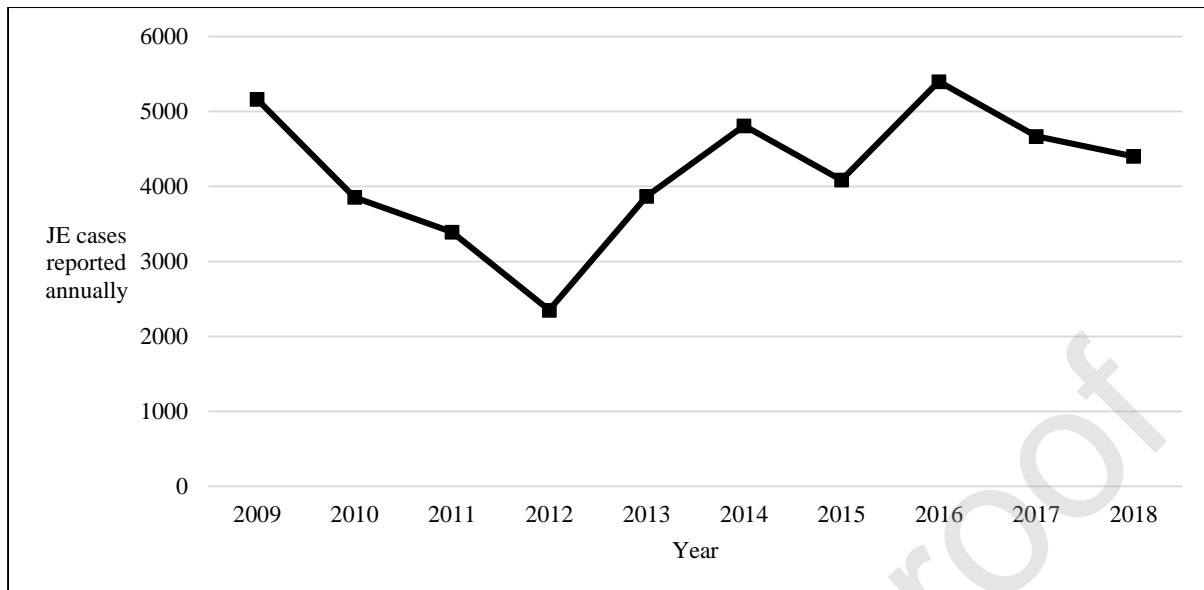
Conclusion: A fundamental pre-requisite for the control of JE is lacking --- that of a simple and specific diagnostic procedure that can be adapted for point-of-care tests and readily used throughout JE endemic regions of the world.

Introduction

The mosquito-borne flavivirus Japanese encephalitis virus (JEV) accounts for an estimated 68,000 cases of Japanese encephalitis and 709,000 disability adjusted life years annually (1, 2). Japanese encephalitis virus (JEV) primarily affects children in rural areas when JEV-infected mosquitoes feed on humans rather than their primary amplifying hosts, pigs or reservoir hosts, i.e. aquatic birds (3). Sustained efforts from international agencies have supported the introduction of immunisation programmes into routine health control schedules in countries with endemic JEV transmission, Table 1 (4). The evidence suggests that vaccination has had an impact on JE incidence (4-10). However, JEV remains a leading cause of neurological infection in endemic countries, and the Joint World Health Organisation (WHO)/United Nations Children's Fund (UNICEF) surveillance data do not substantiate the improvements cited in the past ten years, with sustained numbers of reported patients over this period, see Figure 1.

Table 1: Country-specific data on the introduction of Japanese encephalitis virus vaccine in JE endemic countries. Data adapted from the CDC report by Heffelfinger *et al.* 2017 and updated with WHO surveillance data (4, 11, 12).

Figure 1: Number of JE cases reported annually over the last decade based on WHO/UNICEF surveillance (13). Data include probable* and laboratory confirmed cases reported by JE endemic countries



*WHO definition of a probable case(14) = A case that meets the clinical case definition for acute encephalitis syndrome (AES) that occurs in close geographical and temporal relationship to a laboratory-confirmed case of JE, in the context of an outbreak. Note that these data represent only reported cases, and are not considered to be a true representation of global JE incidence. Weaknesses of these data are discussed in the main text.

JE cases reported to WHO/UNICEF have important limitations. For example, increased awareness of the disease and access to laboratory capacity may contribute to increased case reporting. Conversely, surveillance data are likely to represent only a small proportion of patients (15). This is particularly relevant for JE, occurring predominantly in rural areas lacking diagnostic capacity (16). There are no rapid or point-of-care tests for JE in clinical use (17), and the WHO recommended standard diagnostic assay is an ELISA test that requires trained professionals, appropriate resources and several hours for the results of the tests to be obtained (18). In a survey performed by WHO/UNICEF in 2017, 21 countries responded, of which 11 met the minimum surveillance standards (19, 20). Equally, there are problems of specificity of the most widely used diagnostic test, JE MAC-ELISA (21). This is an increasing issue, with increasing endemicity of other flaviviruses and vaccination coverage.

The reasons for persistence of JE as a public health problem are complex and multifactorial. A key principle that must be kept in mind is that JE is a zoonotic infection, human immunisation will never eradicate it in the natural environment, and therefore sustained vaccination coverage is necessary.

However, in countries that do have vaccination programmes, they are not necessarily uniformly implemented nationwide and in some areas, vaccine coverage is sub-optimal. Whilst there are many reasons for inadequate coverage, this remains a neglected aspect of JE vaccination programmes (22). Furthermore, immunisation strategies are constrained by the absence of adequate diagnostic capacity to investigate the burden of disease, the impact of vaccination (23) and the dynamic epidemiology of JE. For example, in common with other emerging arboviruses (24) JE has the propensity to emerge and become established in new geographical regions (25). In recent years there have been increasingly frequent reports of cases in peri-urban and urban areas (24), as well as new regions such as Rajasthan, India. Moreover, evidence for autochthonous transmission of JEV in Angola was recently reported (26). JEV RNA has also recently been detected in birds in Italy (27). This most likely represents the increased movement globally, via transportation, of animals and goods. Increased pig farming in urban areas of Asian countries also impacts on virus amplification. Finally, the live attenuated vaccine in widespread use is based on JEV genotype 3, despite the fact that in recent decades there has been widespread genotype displacement to genotype 1 (28), and evidence of detection of genotype 5 (29). Accordingly, we aimed to perform a comprehensive review of the evolution of current diagnostic tests for JE. We tackled this by performing a systematic review of published laboratory-confirmed symptomatic cases of JE in humans, and extracted data on the laboratory procedures employed. We also appraised novel tests either under development or conceptually applicable for future diagnostic purposes. Data analysis informed discussion on future perspectives for research.

Methods

Searches were performed in the Web of Science and PubMed using the text word term 'Japanese encephalitis' up to 13th October 2019. The abstracts were reviewed and full text was obtained for those potentially containing information on human cases of JE in the English language. The full text articles were then reviewed for those reporting symptomatic human cases of laboratory-confirmed JE. The search was limited to JE cases confirmed during the acute illness or hospitalisation rather than seroprevalence, with geographic information at least to country of onset of illness, and temporal information at least to year of diagnosis. Data were extracted on details of the diagnostic confirmation of the JE cases. A JE case was classified according to the confirmatory level (1-4) developed from existing WHO and CDC criteria, where 1 provides the highest level of confidence based on the diagnostic test used, as illustrated in Table 2.

Table 2: Diagnostic criteria used to assess JE laboratory confirmed patients*

Results

The review identified 205 studies in 22 countries in which a total of 20,212 JE patients were confirmed by laboratory tests, see Figure 2 for the PRISMA flow diagram. Patients were predominantly diagnosed in Asia, with a suggested case of autochthonous transmission diagnosed in Angola. The studies incorporated a variety of methods for the diagnostic tests, including conventional and novel methods, as summarised in Table 3. The data do not provide evidence of change in the certainty of diagnosis through time, see Table 4.

Figure 2: PRISMA flow diagram

Table 3: Diagnostic methods used for evidence of Japanese encephalitis virus infection

Table 4: Temporal changes in JE diagnostic confirmatory level. Percentage of symptomatic human JE cases reported in the literature in English language in blocks of five years that were confirmed by laboratory testing Level 1-4*.

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Overview of JE diagnostic testing

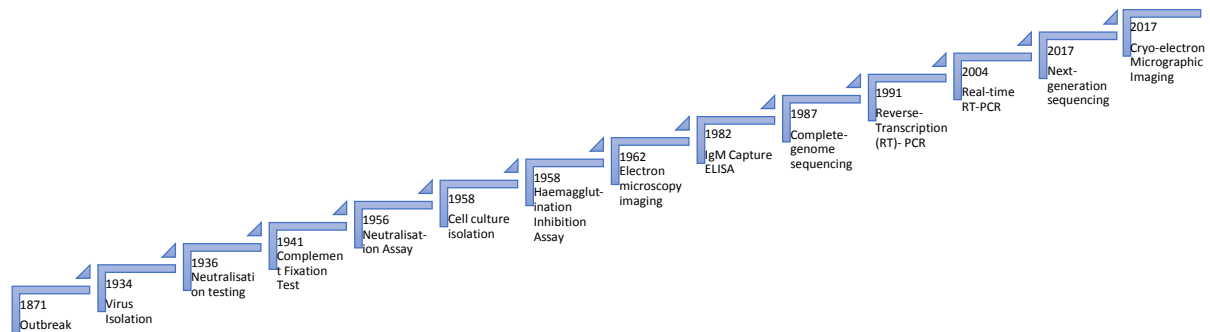
The first isolation of JEV was in 1934, when Hayashi demonstrated that a filterable agent inoculated into monkeys produced encephalitis (31). The experiment was performed using homogenised brain, obtained at post-mortem, from a fatally-infected child who presented with encephalitis in Tokyo during the 1924 epidemic. Early studies relied on clinicopathological correlates in infected humans, when compared with those observed following animal inoculation of post-mortem samples. Subsequently, hamster, porcine, and human cell culture systems were developed which revealed cytopathic effects when inoculated with JEV-infectious specimens (32, 33). As these procedures improved, mosquitoes and mosquito-cell cultures were added to the resources for isolation and identification of JEV (34, 35). Cerebrospinal fluid (CSF) and other body fluids were also included for analysis (36, 37). Subsequently, JEV antigen detection procedures including complement fixation, immunofluorescence microscopy of cells in CSF, reverse passive haemagglutination and staphylococcal coagglutination (38-41) were added to the list of diagnostic tests. Nonetheless, assays involving direct virus detection are minimally useful for the diagnosis of JE as the level of viraemia is usually low and the virus is detectable only briefly early in the infection (42).

In the mid-twentieth century, investigation of the antigenic properties of JEV soon led to the development of serological assays including complement fixation (43), inhibition of haemagglutination (44), and virus neutralisation tests (45, 46). Early reports of human infection in 1947 used a seroneutralisation technique in which a patient sample was mixed with virus and inoculated into mice (45, 47, 48). In 1941, Casals and Palacios published a report on the application of the complement fixation technique (49). The method was used for many years, although it was insensitive, particularly during the acute illness (50). Accordingly, in 1958, Clarke and Casals published a report on the application of the haemagglutination inhibition test (HI) (44). The principle exploits the fact that JEV envelope protein agglutinates erythrocytes. Anti-JEV antibodies, developed following infection, bind to JEV protein and thus prevent erythrocyte agglutination, hence the term haemagglutination-inhibition. This remained the method of choice for JE diagnosis, by serological methods, for many years (51-54) and was subsequently adapted as a more convenient microtiter

method (34, 46, 55). However, limitations in the sensitivity and specificity of the assay were recognised by Clarke and Casals (44). Moreover, the test relies on the combined paired results obtained from acute and convalescent serum samples, thus taking weeks for confirmation (56). Other serological methods such as single-radial haemolysis (57-59) were also introduced. However, inadequacies were readily acknowledged and it was accepted practice to perform these tests in parallel with others, thus increasing the workload and extending the time for results to be obtained (60, 61).

The plaque-reduction neutralisation test (PRNT) was subsequently developed as the gold-standard for JE diagnosis, using paired acute and convalescent sera and comparison with other endemic flaviviruses, and it remains so today (62). The demonstration of increasing anti-JEV neutralising antibody titer in the convalescent serum and the absence or at least fourfold lower titre for neutralising antibodies against control related flaviviruses, provides a robust diagnosis. However this is laborious, time-consuming and requires high level containment facilities for safe manipulation of infectious JEV in cell culture. Largely for these reasons, the anti-JEV IgM capture ELISA (JEV MAC-ELISA) was developed during the 1980s, and has been incorporated as the WHO standard procedure for JE diagnosis (63, 64). Although commercial JEV MAC ELISAs are manufactured they may be hard to access in endemic countries (for example, there is no supplier in Laos) and relatively expensive and required costly ELISA readers and significant technical training. Nevertheless, performance of the kit still requires specialised laboratory equipment and are by no means point-of-care tests. Moreover, field studies suggest that the sensitivity is 50-70% (65), and concerns have been raised regarding the diagnostic specificity (21). In the last two decades, there has been increased availability of molecular testing, providing crucial data on molecular epidemiology. Nonetheless, the aforementioned low and brief viraemia limits the role of testing for JEV RNA for diagnostic purposes. Similarly, advances in techniques such as near-atomic resolution cryo-electron microscopy contribute to our understanding of detailed viral structure, but not to routine detection of human infection.

Figure 4: Chronological representation of discoveries related to the detection of Japanese encephalitis virus infection (32, 46, 48-51, 66, 67)



Specific findings of JEV diagnostics review

Studies reporting on the use of seroneutralisation, IgM ELISA and RT-PCR are discussed below, since these assays are, at present, the ones most widely incorporated into clinical diagnostics.

Seroneutralisation assays: Thirty-two of these included articles identified evidence of JE using neutralisation assays, see Table 5, of which 15 clearly performed tests on acute and convalescent sera, indicating seroconversion (61, 68-83). These were largely PRNT (14 studies), and or a microtitre modification in a 96-well plate (4 studies). Three studies performed focus-reduction seroneutralisation tests (FRNT), a high-throughput modification of the PRNT involving 96-well plates and an immunocalorimetric assay for end-point determination. Other studies did not describe their methods in detail, or cite references to support them. Eleven reported the JEV strain used (45, 61, 77, 86-88, 90-94): they were all genotype 3 viruses, except for one that reported the use of genotype 1 and 3 strains to enable neutralisation-based genotype differentiation (86). Five studies indicated the JEV inoculating dose: The end-point was identified by visual inspection of cytopathic effect (CPE), staining, or immunofluorescence. All reports appeared to use two-fold dilutions of serum samples, between 1:10 to 1:640 or higher. In terms of quality control, 3 studies detailed other viruses used, and the use of replicates. Five studies (45, 61, 88, 91, 93) included the use of other flaviviruses such as dengue viruses, *West Nile virus* or *yellow fever virus*.

Table 5: Details of studies reporting performance of seroneutralisation.

Studies followed different algorithms for including neutralisation in patient testing, but it was largely performed to confirm equivocal cases in other serological tests. Acute and/or follow-up serum and/or CSF were tested. For studies that did report individual results, confirmation was rarely achieved as there was either insufficient serum, failure to detect a four-fold rise of antibody titre in the convalescent serum, or cross-reactivity was detected with related viruses included as controls in the tests.

IgM ELISA: One hundred and sixty-three (80%) studies reported the results of tests using IgM MAC-ELISA methods. Notably, 115 of these studies tested both CSF and serum samples, and presented results for the different body fluids separately. One hundred and twenty-two (74%) reported the method, of which 66 (40%) used in-house methods and 33 (20%) used commercial kits. The main in-house methods involved those described by Burke *et al.* 1985 (63), Innis *et al.* 1989 (95), the National Institute of Virology, Pune (96). Commercial kits were purchased from PanBio (89), Venture Technologies (97), XCyton Diagnostics Ltd. (98) and Shanghai B&C Biological Technology Co. Ltd. (99). There was minimal reporting of quality control measures such as control specimens and repeat testing of positives, and 46 (28%) reported following the manufacturer's instructions. In total, 7,584 JE patients (38%) were diagnosed by MAC-ELISA in serum and/or CSF, i.e. results for the different body fluids were not reported separately, with 3,668 (18%) positive in CSF alone. Ninety-one (56%) studies testing MAC-ELISA also reported testing for Dengue virus infection to confirm specificity for JEV, i.e. they were not cross-reactive with dengue viruses.

Molecular tests: Forty-one studies (25%) reported the use of reverse transcription-polymerase chain reaction tests (RT-PCR), of which 13 (68%) described the methods used or cited corresponding references. These targeted various regions of the JEV genome, including the capsid (C), pre-membrane (prM), envelope (E), non-structural (NS) proteins NS2A, NS3, NS5 and the untranslated

regions (UTR). The studies reported the use of conventional RT-PCR either standard, with nested or hemi-nested techniques, and the real-time techniques (RT-qPCR) using hydrolysis probes or SYBR green. Two studies reported use of commercial kits, and 25 reported use of in-house methods. Most of these studies involved the use of serum and/or CSF, although there were reports of testing other samples such as urine and throat swab samples. In total, 332 (1.7%) patients were positive when tested by RT-PCR.

Other tests: Fifty-eight (28%) studies reported isolation of JEV *in vivo* or *in vitro*. Forty-two (20%) performed HI, 14 (7%) performed complement fixation and 7 (4%) performed indirect immunofluorescence assays. Two (1%) studies diagnosed cases by next-generation sequencing.

Discussion

This review reveals that current JE diagnostic techniques are largely confined to those with a low confidence level, i.e. anti-JEV IgM detected in serum samples, or in which reported results do not differentiate between detection of anti-JEV IgM in CSF and serum. There is no doubt that the introduction of IgM ELISA testing in serum samples represents real progress. However we are now much better informed about the limitations of relying on this method of detection. It is also acknowledged as a limitation that the studies included in the review were performed in different settings, with different constraints, financial limitations, and resources available. Nonetheless, we highlight the need for both the improvement in accuracy of routine laboratory diagnostics, and also the development of point-of-care tests to confirm cases in JE endemic areas frequently devoid of laboratory capacity. Below, we discuss the existing assays in more detail.

Seroneutralisation tests: Seroneutralisation is considered the gold standard for diagnosis of infections due to pathogenic viruses such as JEV, but in the published literature cited herein it was only performed in approximately 16% (32/205) of studies as laboratory confirmation. This is probably due to the fact that performing the seroneutralisation is technically demanding and requires sufficient volumes of serum/CSF to enable the inclusion of control viruses and duplicates of each titration. Since JEV is a human pathogen with high individual risk, seroneutralisation has to be performed in biosafety level 3 laboratory, placing additional burdens on time, cost and qualified personnel. Another potential complication may arise when sera from patients who have previously been exposed to JEV-related flaviviruses may contain higher titres against the closely related flaviviruses than against JEV (“doctrine of original antigenic sin”) (100). For example, the titres of anti-YFV neutralising antibodies were higher than anti-JEV neutralising antibodies in JE patients who had previously received the yellow fever vaccine (101). Similarly, in a study testing West Nile virus and JEV, 18 patients’ data remained equivocal due to high levels of antigenic cross-reactivity between these viruses (91). The neutralisation test may only be strictly applicable as the gold-standard for vaccine efficacy studies, in which a baseline serum sample is compared with a convalescent sample taken at a fixed interval 1-3 months later. For the purpose of confirming acute JEV, neutralisation is an imperfect gold standard.

Severe constraints on being able to arrange for sample testing by neutralisation, and the results being interpretable without cross-reactive positivity due to other flaviviruses (which is relatively rare in JEV endemic areas), impede ‘neutralisation confirmation’. The neutralisation titres obtained may be affected by the particular strain of challenge virus utilised (102). A final issue with the neutralisation test is the inability to detect non-neutralising antibodies, thus potentially reducing the analytical sensitivity (103). Therefore, the practicalities of PRNT and diagnostic yield when testing field samples can be low, although the specificity is potentially high.

IgM ELISA: Anti-JEV IgM detection by MAC-ELISA is the WHO recommended standard diagnostic test, and 80% (163/205) of studies reported the use of a MAC-ELISA. It is recognised that JEV diagnosis by testing CSF provides considerably stronger confirmation than the use of serum (104). However, obtaining acute and convalescent CSF and serum samples can be difficult, particularly in rural Asia where access is logistically difficult and personnel and appropriate facilities are limited. Only 91 (56%) studies reported contemporaneous testing for anti-dengue-specific antibodies. There are issues in the accurate full reporting of results, both for the breakdown of which patients were diagnosed by testing CSF and/or serum, and contemporaneous testing for dengue-specific antibodies.

Reverse-transcription polymerase chain reaction assays (RT-PCR) for detection of JEV RNA:

Diagnosis by the detection of viral genome by DNA amplification generated by RT-PCR is a valuable addition to diagnostic procedures for RNA viruses. The test has high analytical sensitivity, is very specific, and can provide additional information that can be exploited to understand the molecular epidemiology of the detected virus. Nonetheless, JE cases are rarely confirmed (1.7% in this review) using RT-PCR technology, although this will undoubtedly increase in usage as point-of-care and automated methods are developed. Poor reporting of techniques used in many publications hinders the ability to make comparisons of the efficacy of the different methods (see Bharucha *et al.*, 2018) (105). However, there does appear to be higher analytical sensitivity in studies that used nested and hemi-nested techniques as compared with single RT-PCR, but these techniques are notoriously prone to

contamination causing false positive results. It is also recognised that the sensitivity of nucleic acid detection (and protein) detection will continue to increase as technology improves (106, 107). Evidence to suggest that this will be the case arises from the high cycle threshold (Ct) of patients that are confirmed, and the fact that blood donor transmission has been seen in WNV patients who were negative by RT-qPCR tests (108). Recent detection of JEV RNA in throat swabs of JE patients suggests that this non-invasive sample may marginally improve diagnostic yield (109). There have now been two cases of JE, confirmed by RT-PCR, that were first identified by metagenomic next-generation sequencing (mNGS) (110, 111); the first detection of JEV RNA in human urine, and JEV detection in serum from an African patient with a co-YFV infection. The latter was not confirmed by an orthogonal method, and remains questionable. Nonetheless, unbiased mNGS technology (see below), application and reporting will continue to improve, and could potentially detect JEV in novel locations (112).

Requirements of a new test for the detection of JEV infection

CNS infections are challenging syndromes to diagnose and treat, even in the most highly resourced centres (113, 114). It is estimated that they may be caused by >100 different pathogens, including novel and emerging pathogens (115). Current approaches to diagnosis in routine clinical practice involves targeted approaches, suggesting that some (potentially treatable) infectious aetiologies are missed (112). Clinical diagnosis is rarely absolute and confirmation requires access to appropriate laboratory facilities and personnel, lacking in many areas worldwide (116). Whilst analysis of brain biopsy material is the gold standard, it is not possible in most cases. Aetiological diagnosis usually involves an invasive lumbar puncture (LP) to obtain CSF, that requires the appropriate clinical skills, infrastructure and patient acceptability. Diagnostic assays are frequently difficult to interpret, may demonstrate poor accuracy, and poor discrimination between previous vaccination or non-neurological JEV infection (104). Targeted research is needed to raise the bar for both the improvement in laboratory diagnostics as well as development of point-of-care tests (117).

In clinical and epidemiological situations, the detection of JEV RNA can provide an invaluable indication of infection. The sensitivity of this test is to a large extent limited by a combination of the short period of viraemia, the relatively low concentration of virus in CSF and the fragility of RNA. Introduction of highly sensitive point-of-care tests that may be used to analyse multiple body fluids in parallel would partly resolve these challenges. During recent years, there have been significant developments in highly sensitive molecular point-of-care tests for flaviviruses, such as reverse transcription loop-mediated isothermal amplification (RT-LAMP), reverse transcription recombinase polymerase amplification (RT-RPA), nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), helicase-dependent amplification (HDA), and nicking enzyme amplification reaction (NEAR) (118-137). Microfluidics, chips, paper-based devices and biosensors are also being developed (138-142).

For the time being, we will need to rely on serology for diagnostic confirmation. During the past three years, with the international focus on emerging flaviviruses following *Chikungunya virus* and *Zika virus* global epidemics, there have been intensified efforts to reduce cost, increase throughput and improve specificity. These include the analysis of IgA (143-152) and IgG subclasses (146), antibody avidity (143, 153-156), incorporation of blocking agents, IgG depletion (157) and production of specific monoclonal antibodies for identification of specific viral epitopes (158-162). This recent work highlights the inherent challenges of serological techniques for JE identification. As Lindsey *et al.* describe, antigenic cross-reactivity between related viruses can make it virtually impossible to distinguish the cause of the infection (163). For example, cross-reactive IgM class antibodies may not be stimulated during a related secondary flavivirus infection. On the other hand, IgA may be produced during a secondary flavivirus infection and a laboratory-defined 'seroconversion' might be detected following a secondary flavivirus infection by a related flavivirus.

Evidence suggests that the secreted viral JEV non-structural protein 1 (NS1) is present at very low concentrations in serum or CSF, unlike in dengue (164, 165). A novel alternative approach would be to analyse the host response, using transcriptomics or proteomics. However, questions of specificity

and also how these would be translated into point-of-care tests, would require detailed investigation and the development of innovative methodologies.

In summary, whilst the diagnosis of JE has been possible for many years, it still requires specialised high containment laboratories and appropriately trained scientists and therefore cannot be reliably carried out in many resource limited regions where JEV is endemic/epidemic. A fundamental prerequisite in the public health strategy for the control of JE is lacking, that of a reliable and simple diagnostic procedure that can be adapted for point-of-care tests, and readily available for use throughout JEV endemic regions of the world. Improved diagnostic capabilities throughout JEV affected areas will not only benefit individual patients (through accurate diagnosis) but lead to higher quality surveillance data and better understanding of the distribution of JE risk, enabling improved targeting and evaluation of interventions. The lack of diagnostic capabilities for JE is a barrier to understanding the true disease burden and impact of public health strategies.

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Ethical Approval

Ethical approval was not required for this study.

Conflict of interest statement

None of the authors have any conflict of interests to report.

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Figure 2: PRISMA flow diagram illustrating the systematic review process

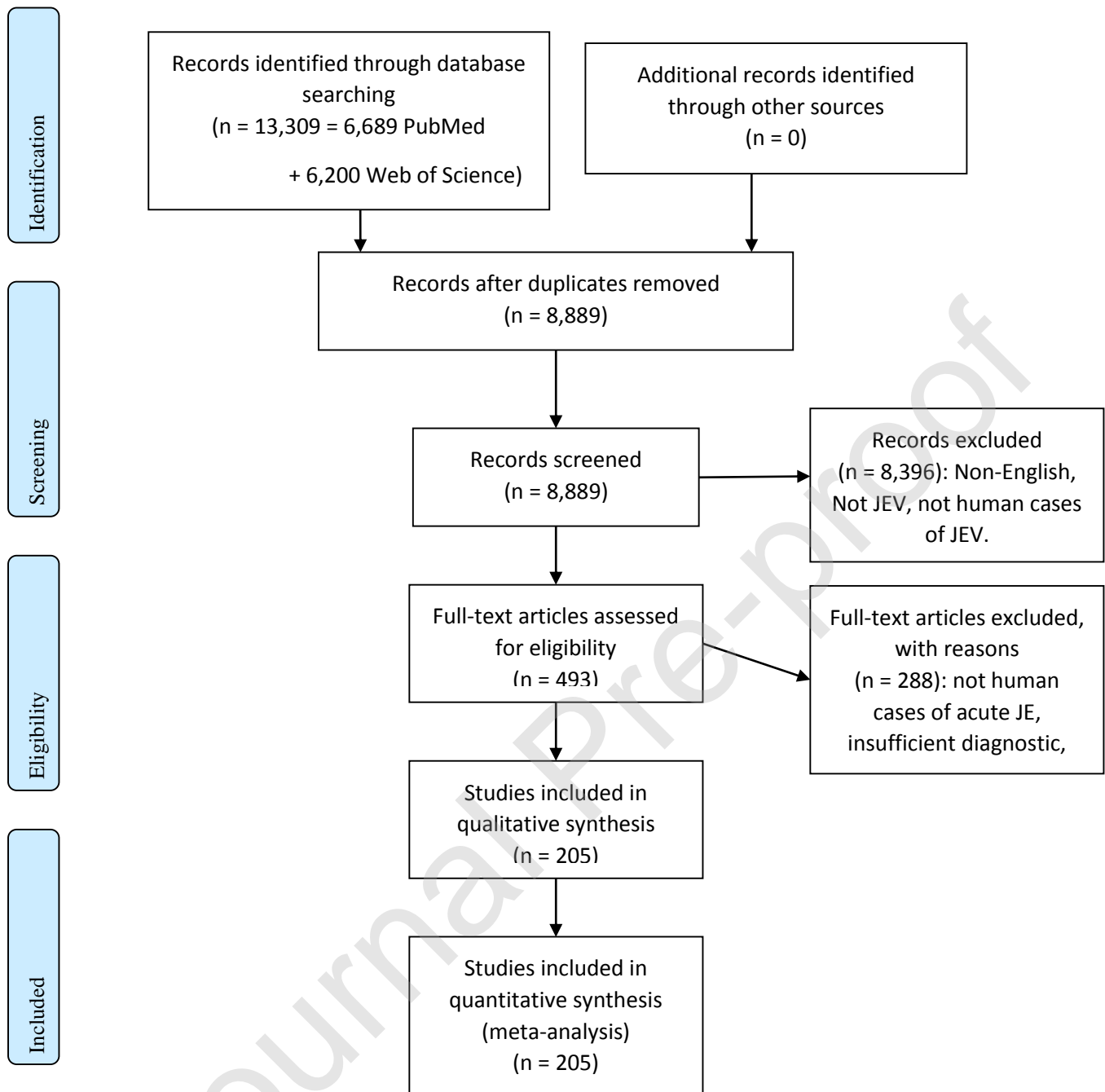


Table 1: Country-specific data on the introduction of Japanese encephalitis virus vaccine in JE endemic countries. Data adapted from the CDC report by Heffelfinger *et al.* 2017 and updated with WHO surveillance data (4, 11, 12).

Country	WHO region	Vaccine in schedule (2019)	JE immunization program	Year introduced subnationally	Year introduced nationally	Scheduled age (months) for vaccine	Vaccine used in immunisation program
Australia	WPRO	Yes	Risk areas: outer islands of Torres Straits	n/a	n/a	12	Live-recombinant
Bangladesh	SEARO	No	None	n/a	n/a	—	—
Bhutan	SEARO	No	None	n/a	n/a	—	—
Brunei Darussalam	WPRO	No	None	n/a	n/a	—	—
Cambodia	WPRO	Yes	National	2009	2015	9	Live-attenuated
People's Republic of China	WPRO	Yes	National; excluding Qinghai, Tibet, Xinjiang, and Hong Kong which do not have endemic transmission	2003*	2008	8	Live-attenuated
DPR of Korea	SEARO	No	None; JEV vaccination campaign in 2016	n/a	n/a	—	—
India	SEARO	Yes	Subnational	2007	n/a	9–11	Live-attenuated
Indonesia	SEARO	Yes	Subnational: Bali	2018	n/a	—	—
Japan	WPRO	Yes	National	<2002	<2007	6	Inactivated vero cell derived
Lao PDR	WPRO	Yes	National	2013	2015	9–11	Live-attenuated
Malaysia	WPRO	Yes	Subnational: Sarawak and Sabah	2002	n/a	9	Live-recombinant
Myanmar	SEARO	Yes	National	n/a	2018	—	—
Nepal	SEARO	Yes	National	2007	2017	12	Live-attenuated
Pakistan	EMRO	No	None	n/a	n/a	—	—
Papua New Guinea	WPRO	No	None	n/a	n/a	—	—
Philippines	WPRO	Yes	Subnational: Regions I–III, and the Cordillera Administrative Region	2018	n/a	—	—
Republic of Korea	WPRO	Yes	National	n/a	<2002	12	Live-attenuated, Live-recombinant, Inactivated vero cell and mouse brain derived
Russian Federation	EURO	No	None	n/a	n/a	—	—
Singapore	WPRO	No	None	n/a	n/a	—	—
Sri Lanka	SEARO	Yes	National	2001	2011	12	Live-attenuated
Republic of China	WPRO	Yes	National	1963	1968	15	Inactivated mouse brain derived
Thailand	SEARO	Yes	National	n/a	<2002	12	Live-attenuated and Live-recombinant
Timor-Leste	SEARO	No	None	n/a	n/a	—	—
Vietnam	WPRO	Yes	National	<2002	2015	12	Inactivated mouse brain derived

*According to official WHO data, although it is acknowledged that the People's Republic of China has performed widespread vaccination since 1971 (166). WPRO = Western Pacific Regional Office; SEARO = South-East Asia Regional Office.

Table 2: Diagnostic criteria used to assess JE laboratory confirmed patients*

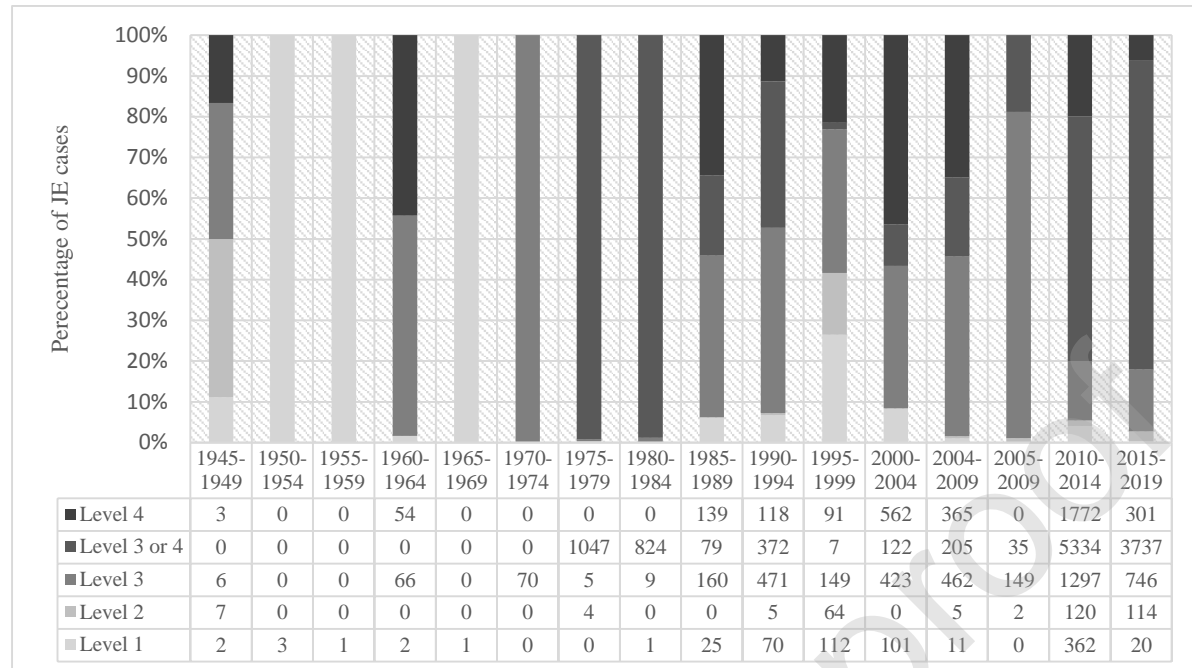
Level 1	JEV RNA detected in any specimen by RT-PCR.
	Virus isolation by inoculation of any specimen in cell culture or animal with characteristic cytopathic effect and confirmation by detection of JEV RNA or virus antigen.
	JEV virus antigen detected from brain tissue or CSF by immunofluorescence or immunohistochemistry
Level 2	Seroconversion or \geq X4 rise in anti-JEV Ab by seroneutralisation or detection of neutralising antibody in CSF; Samples should be tested alongside other endemic flaviviruses (e.g. dengue viruses)
Level 3	Anti-JEV IgM detected in CSF; Samples should be tested alongside other endemic flaviviruses (e.g. dengue viruses)
	Seroconversion or X4 rise in anti-JEV Ab HI, CF, IFA; or seroconversion by ELISA; Samples should be tested alongside other endemic flaviviruses (e.g. dengue viruses).
Level 4	Anti-JEV IgM detected in serum in one sample (acute/convalescent) or seroneutralisation tested in one sample or single high titre HI/CF/IFA; Samples must be tested alongside other endemic flaviviruses (e.g. dengue viruses)

RT-PCR = reverse transcription-polymerase chain reaction; RNA = ribonucleic acid; CSF = cerebrospinal fluid; Ab = antibody; ELISA = enzyme-linked immunosorbent assay; HI = haemagglutination inhibition; CF= complement fixation test; IFA = indirect immunofluorescence assay. *Confirmation of JE is categorised into levels 1-4 based on existing WHO and CDC criteria, such that level 1 provides the highest level of confidence.

Table 3: Diagnostic methods used for evidence of Japanese encephalitis *virus* infection

Diagnostic method	Confirmatory level	Advantage	Disadvantage
<u>Virus detection:</u> Virus Isolation: Inoculation of patient sample into animals Inoculation of patient samples onto primary chick or duck embryo cells, and cell lines including Vero, LLCMK, C6/36, MRC and AP61 Inoculation of patient samples into mosquitoes Virus antigen detection: Reverse passive haemagglutination Immunofluorescence microscopy Staphylococcal coagglutination tests using polyclonal or monoclonal antibodies Monoclonal antibody/immunogold/silver-staining (M-IGSS) ELISA to detect viral protein (NS1)	Level 1	Direct detection of the virus or viral protein and high specificity Viral isolation provides molecular epidemiological data	Low sensitivity, laborious and viral isolation requires biosafety 3 laboratory capacity
<u>Molecular detection:</u> Conventional RT-PCR Real-time RT-PCR Nested PCR Specific vs. pan-flavivirus Multiplex PCR Next-generation sequencing	Level 1	Direct detection of viral genome, provides high specificity and additional molecular epidemiological data	Low sensitivity
<u>Antibody detection:</u> Seroneutralisation IgM antibody capture ELISA Avidin biotin system Biotin labelled immunosorbent assay Nitrocellulose membrane based immunoglobulin M capture dot enzyme immunoassay Haemagglutination inhibition +/- sucrose gradient density centrifugation and 2-mercaptoethanol treatment (2-ME) to detect IgM Complement fixation test Single radial haemolysis	Level 2 for seroconversion demonstrated by neutralisation Level 3 for detection of IgM in CSF and for seroconversion or ≥ 4 x rise in Ab titer; Level 4 for detection of Ab detection in single sample	Good sensitivity Good specificity for primary infection Commercial kit available Good sensitivity	Cross-reaction with other flaviviruses Requires paired samples Labourious Difficult to interpret in secondary infection Limited specificity Cross reaction with other flaviviruses Requires paired samples Difficult to interpret in secondary infection

Table 4: Temporal changes in JE diagnostic confirmatory level. Percentage of symptomatic human JE cases reported in the literature in English language in blocks of five years that were confirmed by laboratory testing Level 1-4*.



* A total of 20,212 laboratory-confirmed JE cases were identified. Data are reported according to year of publication. Inclusion criteria also required geographical (country) and temporal (year) data. Confirmatory levels of JE diagnosis detailed in Table 2, level 1 provides the highest level of confidence. Level 3 or 4 refers to cases that were reported as IgM detected in CSF and/or serum.

Table 5: Details of seroneutralisation testing

Reference	Country sampling	Country testing	Technique	JEV Strain	Other viruses tested	Cells	Virus Dose	End-point	Samples tested	Algorithm for seroneutralisation
Sabin 1947 (47)	Republic of Korea	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal inoculation in mice	NR	NR	Acute and f/up serum, and CSF	All samples
Sabin 1947 (93)	China	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal	NR	NR	Acute and f/up serum, and CSF	All samples
Sabin 1947 (45)	Japan	Japan	Mice inoculation	G3 (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	NR	Intracerebral and intraperitoneal	NR	NR	Acute and f/up serum, and CSF	All samples
Edelman 1973 (167)	Vietnam	Vietnam	PRNT	NR	DENV	NR	NR	NR	NR	NR
Benenson 1975 (168)	Thailand	Thailand	PRNT	G3 strain (Nakayama), human brain, Tokyo, Japan, 1935 (EF571853)	DENV 4	LLC-MK2 cells	50-100 PFU	NR	Acute and f/up serum	NR
Hoke 1988 (169)	Thailand	Thailand	NR	NR	NR	NR	NR	NR	NR	NR

Cardosa 1991 (61)	Malaysia	Malaysia	PRNT	G3 strain (Nakayama), human brai, Tokyo, Japan, 1935 (EF571853)	DENV 2 (16681 strain)	<i>Aedes</i> <i>albopictus</i> C6/36 cells	NR	50%*	Acute and f/up serum	Confirmatory testing after positive MAC- ELISA
Peiris 1992 (68)	Sri Lanka	Sri Lanka	Microtitre VNT	NR	NR	Porcine stable (PS) kidney cells	NR	80%*	Serum (NR if acute and/or f/up)	NR
Wittesjo 1995 (170)	Indonesia	Sweden	PRNT	NR	NR	NR	NR	80%*	Acute and f/up serum	NR
Hennessy 1996 (73)	China	U.S.A.	PRNT	NR	NR	NR	NR	NR	CSF, Acute and f/up serum	All samples
Desai 1997 (171)	India	India	Microtitre VNT	G3 (P20778/P20) human brain, Vellore, India, 1958 (AF080251)	NR	Porcine stable (PS) kidney cells	100 TCID 50	100%*	CSF	All samples
Saito 1999 (101)	Japan	Japan	FRNT	NR	YFV	BHK-21 cells	NR	50%*	CSF, Acute and f/up serum	All samples
Tiroumourougane 2003 (172)	India	India	NR	NR	NR	NR	NR	NR	NR	NR
Cutfield 2005 (173)	China	New Zealand	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR

Hashisaki 2005 (69)	Thailand	U.S.A.	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR
Ompusunggu 2008 (79)	Indonesia	Indonesia	PRNT	NR	NR	NR	NR	NR	Serum (NR if acute and/or convalescent)	NR
Lehtinen 2008 (174)	Thailand	Finland	PRNT	NR	DENV 2	NR	NR	NR	Acute and f/up serum	NR
Ravi 2009 (88)	India	India	PRNT	ChimeriVax™-JEV	ChimeriVax™- DENV 2	Vero cells	NR	NR	CSF	Confirmatory testing after positive or equivocal MAC- ELISA
Touch 2009 (89)	Cambodia	Cambodia	PRNT	NR	NR	Vero cells	NR	NR	NR	NR
Anga 2010 (71)	Papua New Guinea	Australia	PRNT	NR	NR	NR	NR	NR	NR	NR
Hossain 2010 (74)	Bangladesh	U.S.A.	PRNT	NR	NR	NR	NR	90%*	NR	NR
CDC 2010 (70)	U.S.A. (Travellers from the Phillipines)	U.S.A.	NR	NR	NR	NR	NR	NR	CSF	NR

	and Thailand)									
Borah 2011 (94)	India	India	Microtitre VNT	G3 strain (P20778/P20) isolated from human brain in Vellore, India in 1958 (AF080251)	NR	BHK-21 cells	100 TCID50 in 50 µL	50%*	Acute and f/up serum	Patients with paired serum available after MAC-ELISA tested
Lee 2012 (76)	Republic of Korea	Republic of Korea	NR	Not reported	NR	NR	NR	NR	Acute and convalescent serum	Confirmatory testing after positive MAC- ELISA/HI/IIF.
Langevin 2012 (175)	Canada (Traveller from Thailand)	Canada	NR	NR	WNV and DENV	NR	NR	NR	CSF, Acute and convalescent serum	All samples
Hills 2014 (176)	China, Taiwan, Republic of Korea	U.S.A.	NR	NR	NR	NR	NR	NR	Acute and f/up serum	NR
Anukumar 2014 (91)	India	India	Microtitre VNT	G3 (P3) human brain, Bankura, India 1973 (AB379813/Z34095)	WNV	Porcine stable (PS) kidney cells	100 TCID50	50%*	Acute serum	All acute serum

Rayamajhi 2015 (177)	Nepal	U.S.	PRNT	NR	DENV, WNV and Powassan viruses.	NR	NR	NR	NR	Confirmatory testing after positive or equivocal MAC-ELISA
Saito 2015 (86)	Laos	Japan	FRNT	Nakayama (a pathogenic and vaccine strain, Tokyo, Japan, human brain, 1935, G3), Beijing-1 (a pathogenic and vaccine strain, Beijing, China, human brain, 1949, G3), P19-Br (an isolate, Chiang Mai, Thailand, human brain, 1982, G1), LaVS56 (an isolate, Vientiane, Lao PDR, swine sera, 1993, G1), and LaVS145 (an isolate, Vientiane, Lao PDR, swine sera, 1993, G1)	DENV 1 (Hawaiian), 2 (New Guinea B), 3 (H-87), and 4 (H-241) and WNV	BHK-21 cells	NR	50%*	Acute and f/up serum	All samples
Li 2016 (77)	China	China	PRNT	G3 strain (733913) isolated from human brain in Beijing, China 1949 (AY243805/AY243844)	NR	BHK-21 cells	100 PFUs	90%*	Acute and f/up serum	All serum

Sunwoo 2016 (82)	Republic of Korea	Republic of Korea	NR	NR	NR	NR	NR	NR	NR	NR
Kyaw 2019 (92)	Myanmar	Myanmar	FRNT and PRNT	G3 strain (JaOrS982),mosquitos, Japan, 1982 (NC_001437)	DENV 1-4	NR	NR	NR	CSF	NR

G1 and 3 = genotype 1 and 3; NR = not reported; CSF = cerebrospinal fluid; PRNT = plaque reduction neutralisation test; DENV = Dengue virus; VNT = viral neutralisation test; FRNT = focus reduction neutralisation test; TCID = median tissue culture infectious dose. * titer required to reduce dengue viral plaques/focus/CPE by 50%, 80% or 90%. MAC-ELISA = IgM antibody capture enzyme-linked immunosorbent assay, HI = haemagglutination assay, IIF = Indirect immunofluorescence assay.