Flavivirus

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for Flavivirus infection.

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1 Introduction

Flaviviruses are enveloped, spherical positive stranded RNA viruses of 60-70 nm diameter. The genus contains a large number of arthropod-borne viruses that are distributed worldwide. Within Australia the most significant human pathogens have been Murray Valley encephalitis virus (MVEV), dengue virus (DENV) and the Kunjin strain of West Nile virus (KUNV). In February 2022, a cluster of locally acquired infections with Japanese encephalitis virus (JEV) occurred in south-east Australia.

Other flaviviruses uncommonly causing human infection within Australia are Kokobera (KOKV), Alfuy (ALFV), Stratford (STRV) and Edge Hill (EHV), although only KOKV has been shown to cause human disease. A number of other flaviviruses have been found in animals or insects within Australia, but none have been shown to infect humans. Most are endemic to the tropical areas of Australia.All of the flaviviruses found in Australia are mosquito-borne and most are maintained in a mosquito-animal cycle, with humans as incidental hosts. The exception is DENV, for which a human-mosquito cycle maintain activity during epidemics.

DENV is widespread in south Asia, sub-Saharan Africa, and Central America. It is not currently endemic to Australia, but is regularly reintroduced into northern Queensland from endemic areas such as Indonesia, Papua New Guinea, and Thailand, producing limited local spread and periodic epidemics[1]. There are 4 serotypes of DENV (serotypes 1-4). Infection with one serotype is thought to confer lifelong type-specific immunity, but only short-lived cross-immunity between serotypes, thereby allowing the possibility of secondary dengue infection with a heterologous serotype[2]. DENV was absent from Australia between 1955 and 1981. Since then there have been numerous large outbreaks of various serotypes in northern Queensland due to transient introductions: DENV-1 in 1981-82 and 1990-91; DENV-2 in 1992/93; and DENV-3 in 1997-99 and 2008-09. Since 2005, outbreaks due to each of the 4 DENV serotypes have been documented[1]. The primary vector for DENV is

the *Aedes aegypti* mosquito, which is present in northern Queensland, but other vector species occur, including *Aedes albopictus*, which is found in the northern Torres Strait Islands. Rare sporadic cases acquired within mainland northern Australia but outside Queensland have been reported in recent years, and postulated to be due to the introduction of an infected *Ae. aegypti* mosquito in air cargo[3,4]. They have not led to any further establishment of the vector or the virus in those areas.

DENV causes a characteristic clinical illness with fever, retro-orbital pain and headache, myalgia, arthralgia, and rash. Thrombocytopaenia and leucopaenia are common. In most cases, dengue is a self-limiting illness, but more severe forms carry a substantial mortality. In 2009, the World Health Organization (WHO) re-classified dengue disease into levels of severity[5]. Severe dengue, which includes dengue haemorrhagic fever and dengue shock syndrome, is defined by presence of severe plasma leakage, severe bleeding, or severe organ involvement and, in the latter case, with shock secondary to plasma leakage. Severe dengue is more commonly found in children and in secondary dengue infection. The exact pathophysiology of severe dengue in secondary infection remains unclear but is thought to be due to antibody-dependent enhancement of viral replication during secondary infection with a heterologous serotype.

MVEV is endemic in the Kimberley region of Western Australia (WA) and the Top End of the Northern Territory (NT), where it is maintained in enzootic foci, primarily in a cycle between water birds and *Culex annulirostris*[6], the major vector for MVEV as well as KUNV and JEV[7]. Activity outside the endemic areas is dependent on heavy summer rainfall with flooding, sufficient to allow migration of infected waterbirds from these areas, usually in a southerly direction within WA and the NT, and eastwards into northern Queensland. There were also several outbreaks of MVEV encephalitis on the east coast of Australia during the 20th century, the latest occurring in 1974 with 58 recognised cases[6]. Between 1975 and 2010, apart from several cases in central Australia in 2000 following heavy rainfall[8] and one case in NSW in 2008, MVEV encephalitis was confined to endemic and adjacent areas. An unusually high number of MVEV cases were seen across Australia in 2011, predominantly in WA and NT, but including several in NSW and SA. Most people who are infected with MVEV will be asymptomatic or have a non-specific febrile illness, sometimes with headache. Encephalitis develops in 1:150 to 1:1000 infected individuals and may be more frequent in children than in adults. Mortality of encephalitic MVEV is reported to be 15-30%, with long-term neurological sequelae developing in about 30-50% of survivors. Deaths and severe sequelae occur largely in young children and older adults.

JEV causes encephalitic disease in infected individuals which is clinically similar to MVEV. In February 2022 a cluster of cases was detected in South East Australia, following detection of JEV in stillborn pigs. At time of writing there have been cases detected in Victoria, New South Wales, South Australia and Queensland. JEV is divided into five genotypes based on typing of the viral envelope (E) gene. Genotypes 1 and 3 have caused the majority of cases reported worldwide, but the current Australian cases are of genotype 4, which is uncommon and previously limited to Indonesia and Papua New Guinea. JEV has also been regularly found in the Torres Strait Islands and mainland Australia at Cape York.

Humans are dead-end hosts for JEV. Amplification occurs primarily in pigs, which typically demonstrate high-level viraemia and act as a reservoir of infection in endemic areas. Infected piggeries have been identified in Victoria, New South Wales, Queensland and Victoria. There has also

been detection of JEV in feral pigs in the NT. It is therefore likely that JEV will become endemic in Australia, but public health efforts are underway to contain this. There are several vaccines against JEV available.[9]

Overseas acquisition of other flaviviruses also occurs. For example, cases of DENV occurring in Australia are most commonly found in returned travellers, particular those returning from Indonesia[7]. Yellow fever virus (YFV) is not present in Australia but may be seen occasionally in people arriving from overseas or post-vaccination disease may occur.

KUNV is a clade (1b) of West Nile virus (WNV) and is found in Australia and Oceania. Of the other clades of WNV lineage 1, 1a is found in Europe, North America, the Middle East, northern Africa, and central and western Asia, while 1c is found in India. The other major WNV lineage (lineage 2) is confined to Africa. KUNV appears to have a similar distribution and ecology to MVEV, with the exception that activity has occurred occasionally but regularly in South East Australia since 1974. It is responsible for about 5-10% of the cases of arboviral encephalitis in Australia, and otherwise may be asymptomatic or cause a mild febrile illness sometimes accompanied by headache, rash and arthralgia. In a 2011 review[10] no fatalities were reported and, although numbers were small, it appeared that neurological sequelae were milder than those for MVEV and 1a clade of WNV.

Zika (ZIKV) virus was first identified in rhesus monkeys in Africa in 1947 in the Zika Forest in Uganda. It was first isolated from a human in the early 1950s and has circulated in Africa ever since. It was first identified in Asia in 1966 in Malaysia, which was a distinct lineage compared with the African strains. The virus has remained in that region since then, though large outbreaks have not been noted. Until 2007, ZIKV was confined to Africa and south Asia[11], but in 2007 there was an entry from Asia that caused a large outbreak in Yap Island in Micronesia, with a population infection rate of over 70%[12]. The next large outbreak occurred in French Polynesia in 2011 and New Caledonia in 2012, due to a separate episode of spread of an Asian lineage strain. It is unclear how long ZIKV has circulated in the Asia-Pacific prior to that, but probably for many years at least. Following the spread through Melanesia and Polynesia, the virus spread via Easter Island to mainland South America in 2015. There have been subsequent large outbreaks in many countries of Central and South America and the Caribbean[12,13]. South American strains have also spread to Cabo Verde off the west Coast of Africa, Mexico and Florida. It is maintained in a human-mosquito-human cycle, though sylvatic cycles still remain. Spread is primarily by *Ae. aegypti*, but spread has also occurred via *Ae. albopictus*, *Ae. hensilii*, and *Ae. polynesiensis*. It can infect a range of other species, but none have yet been shown to be important in the spread of human disease.

The disease caused by ZIKV is generally mild, with fever, a generalised itchy rash, conjunctivitis and joint pains, similar to DENV but milder. This illness is self-limiting, though Guillain-Barre syndrome has been seen following a small percentage of cases. Infection may be asymptomatic, but it is not yet clear how common that is. The main impact has related to the increase of microcephaly, other congenital anomalies, fetal growth retardation, and fetal deaths. Several cases of ZIKV infection acquired overseas have been identified in Australia since 2013[14]. There has been no locally acquired disease in Australia, though northern Queensland is potentially receptive to its entry and circulation.

Specific tests for ZIKV and YFV are not widely available outside reference laboratories, and serology may be difficult to interpret, with cross-reactivity from one of the more common flaviviruses, or IgM not

being detected due to the absence of a specific test. It is important that laboratories bear this in mind when ZIKV is suspected and refer the samples for specific tests if necessary. Interpretation of serology may be challenging in persons previously vaccinated against JEV or YFV, where antibody responses to any infecting flavivirus may be attenuated or post-vaccination responses may be misinterpreted as indicating recent infection. YFV IgM generally develops four to seven days post vaccination, is detectable in 90-100% of recipients by 30 days, and can persist up to three to four years post-vaccination[15].

The ecology of KOKV is not well understood, and appears to more closely resemble that of the alphavirus Ross River virus than that of the other flaviviruses. Human infection has been demonstrated and it has been shown to cause polyarthralgia in a handful of patients.

2. Overview of Laboratory Diagnostic Tests for Flaviviruses

2.1 Culture

Flaviviruses can be detected by cell culture techniques via inoculation of clinical samples into appropriate cell lines. Where culture is attempted, the specimen is typically inoculated on to a mosquito cell line (C6/36 is most commonly used) and incubated for 3-4 days at 28oC. Cytopathic effect is generally absent in mosquito cell lines, so growth is usually identified by the binding of specific monoclonal antibodies, neutralisation with specific antisera, or by specific nucleic acid amplification tests (NAATs). Success of viral culture varies by flavivirus and the cell lines used. Usually virus can only be recovered within the first few days of illness prior to the appearance of antibody.

2.2 RNA detection by nucleic acid testing

A number of studies have looked at the application of NAATs for detection of flaviviruses, either using flavivirus universal primers, or primers targeted at sequences specific for individual viruses (refer below for specific flaviviruses).

2.3 Serological tests

2.3.1 Antigen detection tests

The only widely available flavivirus antigen detection test is for dengue, targeting the NS1 antigen (refer below). Antigen detection tests for CHIKV have also been described[16]. Fluorescent antibodies and immunohistochemistry have been used to identify flavivirus antibodies in tissues but are not routinely used in acute diagnosis[5].

2.3.2 Antibody detection tests

These tests are generally used outside reference laboratories for the diagnosis of flavivirus infections other than dengue. Flaviviruses evoke antibodies that are widely cross-reactive within the genus. This causes challenges in characterising antibody to the level required to identify the infecting virus. Furthermore, due to antigenic relatedness, infection with a second flavivirus (e.g. MVEV infection in

someone with past KUNV) may cause a misleading rise of antibody to the previously encountered flavivirus[17].

In typical cases of DENV infection, laboratory characterisation of the antibody is usually unnecessary due to the confidence of clinical diagnosis[18]. However for other illnesses that are either clinically difficult to differentiate and/or of major public health significance, full characterisation of the antibodies that are produced is desirable.

IgM usually appears within a few days of onset of illness in primary infection. A negative IgM using a sensitive test such as EIA or IFA in a sample collected a week or more into the illness makes recent infection unlikely. For samples collected earlier in the illness or where there is a strong clinical suspicion despite the negative IgM, a second sample at least 2 weeks after onset is recommended. False positive IgM results may occur, therefore acute and convalescent samples should be obtained wherever possible to test for IgG seroconversion or a significant rise in antibody levels or titres. This is particularly important for unusual clinical infections, infections of high public health significance, and where an infection has occurred outside an area of known current activity. IgM is often absent in secondary dengue infections and less commonly, this may occur when the patient has had a different flavivirus infection or vaccination in the past, e.g. acute MVEV infection in someone with past KUNV infection.

Serology results should always be interpreted in light of the accompanying clinical, epidemiological and vaccination history.

2.3.2.1 Suitable Specimens

Serum from clotted blood is commonly used for serological testing, but heparin or EDTA plasma may be suitable if the test is validated for these specimen types. For commercial tests, the manufacturer's instructions should be consulted.

2.3.2.2 Enzyme Immunoassay

Commercial kits are available for the detection of IgG and IgM antibody to certain flaviviruses by EIA, and some laboratories may have in-house EIAs. Changes in optical density between acute and convalescent samples should not be used to measure rises in antibody level unless the test has been specifically validated for the purpose.

2.3.2.3 Immunofluorescent Antibody (IFA)

IFA assays can be developed to detect IgG, IgM or IgA antibody to any of the flaviviruses. Antigen is prepared by growing the flavivirus in a receptive cell line. Infected cells are fixed onto a glass slide and used for a standard indirect IFA. For IgM detection, it is essential that rheumatoid factor and IgG be removed from the sample before testing to avoid false positives and false negatives respectively. Commercial indirect IFA slides are now available for detection of IgM directed against a number of flaviviruses, including DENV, JEV, WNV, YFV and ZIKV.

2.3.2.4 Haemagglutination Inhibition (HI)

The flaviviruses possess an antigen (hemagglutinin or HA) that will agglutinate goose red cells within a narrow pH range. Antibody to the HA will inhibit its activity, which is the basis of the HI titre. The HI test is used less frequently than EIAs or IFAs.

2.3.2.5 Neutralisation Titres

Traditional neutralisation titres may be performed in reference laboratories and measure the ability of the patient's serum to stop replication of the virus by binding to surface antigens and preventing virus uptake. There are several methods that can be used to show inhibition of growth, and these may yield slightly different titres. Microneutralisation assays are used most commonly, where the endpoint is the dilution that inhibits growth by 50% (TCID50). These assays may be less sensitive and specific than conventional plaque reduction neutralisation titres (PRNT). PRNT endpoints can be read at 50% or 90% inhibition, and the latter may be more specific. Other methods use a monoclonal antibody with a dye-attached to detect infected cell (focus-reduction neutralisation titre or FRNT) and NAAT-based methods have been developed for some. Any of these can be used provided that an appropriate range of viruses is tested, and proper criteria are used for interpretation (refer below).

2.3.2.6 Other serologic tests

Other research or reference tests include improved monoclonal antibody epitope-blocking EIAs. These are potentially useful for improving the accuracy of serological diagnosis. For example, a number of commercial epitope-blocking EIAs for WNV, MVE and KUN have been described for use on human and non-human serum for surveillance and diagnostic purposes[19-21]. These assays are much simpler and quicker to perform than plaque reduction neutralisation assays, but current epitope-blocking assays for flaviviruses commonly seen in Australia are in-house assays restricted to reference laboratories. Other developments focus on identification of specific epitopes that may be used in conventional EIA formats. IgG avidity assays have been evaluated for some flaviviruses, but are not in common use and interpretive criteria are not yet available for them.

Laboratory diagnosis of specific flavivirus infections

2.4 Laboratory diagnosis of Zika virus infection

2.4.1. Nucleic acid detection

ZIKV NAAT has proven useful in the confirmation of cases in returned travellers[14,22]. ZIKV can be detected in a number of body fluids, including blood, urine, semen, genital tract secretions and saliva, as well as amniotic fluid, placenta and foetal tissues in congenital infection. ZIKV-RNA load in the urine has been found to be higher than blood levels in some studies, and to persist for longer. Viral RNA may be detectable in semen for weeks or months[23].

ZIKV can be detected in blood and saliva from the time of onset of illness for about a week. It persists in urine for approximately another seven days[24] and has been found in semen up to six months after onset of illness[25]. Levels of RNA are higher and persist for longer in whole blood than serum/plasma, and possibly longer than in urine[26]. Viruses may also be found in placental and foetal tissues and amniotic fluid in congenital infections. Dried blood spots have been used for detection of ZIKV RNA.

RNA can be detected by a range of in-house NAATs directed at different targets, including the NS5 and E genes.

2.4.2 Serological diagnosis

Serological responses to ZIKV are similar to the other flaviviruses. Following primary infection IgM appears 5-7 days after onset of illness and persists for several weeks or months. It is cross-reactive across a range of other flaviviruses. IgG appears shortly after IgM and rises over 1-2 weeks. Rising titres between acute and convalescent sera confirm recent infection. Due to cross-reactivity with other flaviviruses antibody specificity by neutralization titres or another specific serology assay should be performed. To improve specificity, plaque or focus reduction assay with a >90% inhibition endpoint is recommended[23]. The titre on this assay should be shown to be at least 1:40 and to be at least four-fold higher than the titre to any other likely infecting flaviviruses.

2.5 Laboratory diagnosis of JEV infection

2.5.1 Nucleic acid detection

The viraemia caused by JEV is typically low-level and of short duration. As such, nucleic acid amplification techniques are often negative. Positivity rates vary by test population but are typically in the rage of 0 – 30%[27].

There are no commercially available JEV PCR assays. In-house assays have been described targeting conserved areas of the viral genome including non-structural (NS) proteins and the E gene. These assays may be JEV-specific, or amplify conserved flavivirus targets which requires sequencing of the product to confirm the infecting virus[28]. Most NAAT assays have been developed using JEV sequences from genotypes 1 and 3 which have historically caused most infections. Laboratories should be aware of that the analytical performance of some NAAT assays is reduced for the detection of genotype 4 due to primer and/or probe mismatches, and should validate their in-house assays accordingly.

Cerebrospinal fluid is the preferred sample type for detection. Whole blood and urine are also recommended based on previously reported detection in these sample types[29]. The level of virus in serum and plasma samples is generally low. NAAT of serum has been described with a high positivity rate in one case series[30], but this has not been replicated in other studies[27]. Throat swabs have also been evaluated in one study in a high prevalence setting[31], but there is insufficient evidence to recommend routine testing via this method at present.

Loop-mediated isothermal amplification (LAMP)-based methods have been developed and may be suitable for use in resource-limited settings[32], however there is limited data on their use is a low prevalence setting. In addition, LAMP-based assays are generally less sensitive compared with conventional reverse transcription polymerase chain reaction (RT-PCR) assays when viral loads are low.

Where JEV is not suspected to be the cause of encephalitis, metagenomic next generation sequencing (mNGS) approaches (for example RNA-based mNGS) may be useful to identify the causative pathogen. mNGS is not widely available at present, but is anticipated to be used more often

in the near future. Sample quality, storage and transport is key to optimising the diagnostic yield of mNGS. The detection of JEV by mNGS should be confirmed by an alternate NAAT such as RT-PCR assays.

2.5.2 Serological diagnosis

The mainstay of diagnosis of JEV causing encephalitic illness is detection of IgM to JEV in CSF, which is the WHO recommended method of diagnosis[33].

The sensitivity and specificity of JEV-specific IgM detection in CSF is high in most reported studies. It should be noted that in many of these, IgM detection in CSF is considered the gold standard diagnostic modality, and as such may over-estimate the sensitivity. Detection of IgM was found to be 84-88% sensitive in acute JE using an IgM capture EIA[34-36], and a dot EIA was 73% sensitive for samples drawn at hospital admission[37]. Specificity is typically >95%.

Antibody dynamics were described in a study by Han *et al*[38] which found IgM in serum in 75% of cases within 4 days of onset. False negatives may occur if CSF is obtained and tested in the early phases of illness. IgM has been shown to persist for up to 90 days. JEV IgG in CSF and serum peaks later than IgM. Seropositivity in CSF is reported to be 47% on day one of presentation but increased to 100% by day 30. In the same study, seropositivity to IgG in serum was lower at 20% at illness onset, peaking to 100% by day 30[39].

There are a number of commercially available JEV antibody assays including immunofluorescence[40] and enzyme immunoassays[41]. Specificity of these assays is high, but reported sensitivities are lower than comparator methods.

2.6 Laboratory diagnosis of Dengue infection

The antibody response to DENV infection is affected by previous exposure to flaviviruses. In those with no previous flavivirus infection or vaccination, the primary antibody response is generally slower. IgM antibodies appear first, detectable in 50% by day three to five after symptom onset, increasing to 80% by day five and 99% by day ten. IgM then declines to undetectable levels by two to three months. IgG levels are generally detectable one week after onset and continue to increase slowly, peaking later than IgM. IgG levels are detectable for at least several months, probably for life.

In secondary dengue infection, antibody titres rise rapidly, with a predominantly IgG response. This IgG is heterotypic, i.e. it is an anamnestic response directed at the previously infecting serotype. A similar response may be seen in individuals with previous infection by a different flavivirus or with previous flavivirus vaccination. In secondary dengue, IgM levels are significantly lower than in primary infection and may be undetectable.

Differences in antibody responses have been used to differentiate primary and secondary dengue in the laboratory. As HI primarily measures IgG, high HI titres (>1:1280) are traditionally considered indicative of secondary infection, as levels up to 1:1280 may be found in primary infection[42]. However, this rapid anamnestic flavivirus response may also occur in primary dengue if the person has had previous exposure to another flavivirus.

More recently, IgM/IgG ratios have been used as a more specific method of differentiating primary and secondary dengue[43]. To determine the ratio, the patient's serum is first diluted 1:20 or 1:100, then IgM and IgG EIAs are performed. If the ratio of the raw optical densities (OD) of the IgM and IgG EIA is <1.4 (for the 1:20 dilution) or <1.2 (for the 1:100 dilution), this is indicative of secondary dengue infection. As IgG levels eventually come to predominate during convalescence following primary dengue infection, using IgM/IgG ratios to determine secondary infection is generally performed only on acute sera collected within 30 days of symptom onset[44]. Furthermore, the use of IgM/IgG ratios to classify dengue infection has not been standardised across tests, and any IgM/IgG EIA tests not validated for this purpose would require in-house validation[43].

IgG avidity has also been used to distinguish primary from secondary dengue. A study published in 2011 looked at IgG avidity in convalescent sera from primary and secondary dengue cases collected within 37 days of symptom onset[44]. IgG avidity of 0.39 was determined as the cut-off, with 95% of primary infections having avidity ≤0.39 and 95% of secondary cases having avidity >0.39. As with IgM/IgG ratios, use of avidity to distinguish primary and secondary dengue infection has not been standardised across tests.

High HI, low IgM/IgG ratio, and high avidity are discussed in the WHO 2009 diagnostic guidelines for dengue as suggestive of secondary infection[5]. The Centers for Disease Control and Prevention classify dengue infection as secondary if IgG is detected during the acute phase (first days of illness) and there is a four-fold or greater rise in IgG titre in serum collected during the convalescent phase[45].

As DENV is not currently endemic in Australia and the majority of DENV cases are in returned travellers, there is currently a low pre-test probability of secondary dengue infection in Australia compared to areas with high endemicity. For this reason, the laboratory techniques used to differentiate primary and secondary dengue worldwide are of limited value in the Australian context, but this may change as larger numbers of Australian residents and visitors may have had past flavivirus infection or vaccination (noting that there are many Australians born, or have spent a considerable amount of time, in DENV endemic countries).

2.6.1. Nucleic acid detection

Since the 1990s, numerous studies evaluating NAAT such as RT-PCR for DENV have been published, for detection of viral RNA in clinical specimens and in mosquitoes[5]. The 3'-UTR is a commonly used target, while NS5 and C/PrM gene targets have also been used[46,47]. The main advantage of dengue RT-PCR is better sensitivity compared to culture and a more rapid turnaround time. Another advantage is that serotype-specific RT-PCR can be used to perform typing more reliably and more quickly than by serology.

In a large study in children[48], dengue RT-PCR was found to be about 92% sensitive (compared with culture and serology) and 96% specific. The sensitivity of dengue RT-PCR varies by time from onset of illness. A study published in 2014 reported sensitivities of a real-time RT-PCR as 75% on day 1, 92.9% on day three, falling to 50% by day five[49]. In a study performed in WA from an outbreak in Timor, approximately 80% of early samples (HI titre <10) were PCR positive, and about 60% of later samples (DW Smith, personal communication). RNA is also detectable by RT-PCR early in the febrile phase of illness in secondary dengue[50], though the duration is shorter and the viral load is lower than in primary infection, independent of development of severe dengue[51]. Secondary dengue was also

associated with a shorter time to defervescence, so RNA detection is greatest early in the acute phase of the illness. Further, the duration of viraemia corresponds well with the duration of NS1 antigenaemia in both primary and secondary dengue infection[51], though RT-PCR is more sensitive than antigen detection in secondary cases.

Many of the published RT-PCR assays are developed in-house, though a number of commercial assays are now available, including on rapid RT-PCR platforms[52]. A 2014 study evaluated four commercially available RT-PCR assays compared to an in-house hemi-nested RT-PCR[53]. Two offered quantitative results, one offered serotype-specific results. Reported sensitivities were 83-93%, but this varied for serotypes, with one of the quantitative assays lacking sensitivity for detection of DENV-4.

2.6.2 Dengue NS1 antigen test

The first enzyme immunoassay to detect the DENV NS1 antigen in serum was developed in 2000. The test has now become widespread for use in diagnosis of DENV[54], with numerous commercial kits available. Commercial NS1 antigen detection tests do not differentiate between dengue serotypes. NS1 antigen is generally detectable early in the acute phase of primary and secondary infection. In primary dengue infection, NS1 is usually detectable several days before antibody. Detection rate is inversely proportional to the presence of IgG antibodies though NS1 has been detected up to day 14 after onset of illness[55].

As with viraemia, the duration of antigenaemia is significantly shorter in secondary dengue than in primary[51]. This is thought to be due to rapid production of anti-NS1 IgG in secondary infection, causing formation of immune complexes that remove NS1 from the circulation or interfere with the ability of assays to detect it. The diagnostic sensitivity of NS1 antigen for secondary dengue is therefore lower than for primary dengue[55]. Sensitivity of NS1 by EIA is estimated at 90% during the febrile phase in primary dengue infection, and 60-80% during the febrile phase in secondary infection[2].

Dengue NS1 antigen appears to be highly specific for DENV though cross-reactivity studies are limited[56]. False-positives have been reported. For example, a Vietnamese study published in 2009 tested 459 patients with acute fever[57]. Two patients were reported as having false-positive NS1 EIA tests, one of which was strongly positive.

2.7 Laboratory diagnosis of other flavivirus infections

2.7.1 Nucleic acid detection

MVEV-RNA has been detected in serum[17] and CSF[58] of cases. However, these assays are currently limited to reference laboratories.

2.7.2 Serological diagnoses

The sensitivities of IgM assays for other flaviviruses have not been established. Data from the MVEV and KUNV cases in WA and the NT in 2000/01 showed the indirect IFA IgM to be relatively specific, i.e. most patients have IgM only to the infecting flavivirus. In 25 serum samples from MVEV cases with positive MVEV IgM, only six also had a KUNV IgM detected and in all cases the reactivity was weaker than the MVEV IgM. For eight samples from KUNV cases with positive IgM, one had a weak MVEV IgM detected also. CSF IgM was specific for the infecting virus, but data are limited.

3 External Quality Assurance

A WHO QAP for dengue diagnostics including specimens for detection of NS1 and RNA, and IgM and IgG antibodies, was introduced for the WHO Western Pacific Region in 2013[59]. In addition, a RCPA QAP is available for flaviviruses.

4 PHLN Laboratory Definitions

4.1 Condition

4.1.1 Recent flavivirus infection – common diagnostic criteria

Definitive Criteria

- Isolation of flavivirus from clinical material; OR
- Detection of flavivirus-specific RNA in clinical material; OR
- Seroconversion or significant increase in IgG level or titre to a flavivirus.
    - Significant increase is defined by the testing laboratory based on test characteristics of assays used and could include rising titre (if measured by IFA or other assays where titres are available).
    - Specificity of flavivirus antibody testing may be improved by measuring antibodies using two assays which utilise different methodologies (e.g. IFA and EIA) OR detect antibodies against different viral epitopes.
    - Cross-reactivity of antibodies against flaviviruses is common. Antibodies against other clinically compatible and epidemiologically related flaviviruses should be performed to aid interpretation of likely cause of IgG rise.
        - Where MVEV, KUNV or JEV infection is suspected, serological tests should be done for antibody to DENV, MVEV, KUNV and JEV as a minimum.
        - Where ZIKV infection is suspected, serological tests should be done for ZIKV and DENV as a minimum.
        - Where YFV infection is suspected, serological tests should be done for YFV, KUNV, DENV, JEV and ZIKV as a minimum.
        - If substantial cross-reactivity limits interpretation of likely infecting virus, assays with higher specificity such as epitope blocking assays or viral neutralisation tests can be considered.

Suggestive Criteria

- Detection of IgM to a single flavivirus.
    - Wherever possible, acute and convalescent samples should be collected to confirm recent infection
    - A specific virus can be assigned if the IgM to a single flavivirus is detected in the absence of IgM to other likely flaviviruses provided that there is a suitable clinical and

exposure history. Unspecified flavivirus infection if IgM is detected against more than one virus or the full range of flaviviruses has not been tested, provided that there is a suitable clinical and exposure history.

- Detection of single IgG to a flavivirus in individual with compatible epidemiology and clinical syndrome
    - Serial serology to assess for change in level of IgG should be considered.
- Detection of IgG to a single flavivirus in an individual with compatible epidemiology and clinical syndrome without significant change in level of IgG may be considered if initial sample was taken late in clinical illness.

For all suggestive criteria, cross-reactivity of antibodies against flaviviruses is common. Antibodies against other epidemiologically appropriate flaviviruses should be performed as described above to aid interpretation of likely cause of antibodies detected.

4.1.2 Recent dengue virus infection

Definitive Criteria

Isolation of dengue virus from clinical material; OR- Detection of dengue viral RNA in clinical material; OR- Detection of dengue NS1 antigen in serum or plasma; OR - Seroconversion or significant increase in dengue IgG level or titre in serum or plasma provided the IgG is shown to be specific to dengue virus by neutralisation or other specific tests.

Suggestive Criteria

Detection of IgM to dengue virus.

4.1.3 Secondary dengue virus infection

Definitive Criteria

Fulfilment of definitive criteria for recent dengue infection in a person with previous laboratory confirmed dengue infection.

Suggestive Criteria

- Dengue HI titre ≥1:2560; OR
- Dengue IgM/IgG OD ratio <1.4 when tested at 1:20 dilution or <1.2 when tested at 1:100 dilution of serum collected within 30 days of symptom onset; OR
- Detection of dengue IgG within 7 days of symptom onset with an accompanying four-fold rise in dengue IgG titre during convalescence.

4.1.4 Recent flavivirus infection

Definitive Criteria

- Isolation of flavivirus from clinical material; OR
- Detection of flavivirus-specific RNA in clinical material; OR
- Seroconversion or significant increase in IgG level or titre to a flavivirus. A specific viral diagnosis can be assigned if the IgG is shown to be specific to a single virus, by neutralisation or other specific tests. Unspecified flavivirus infection if the IgG cannot be shown to be specific to a single virus.

5. References

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