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Title: **FLAVIVIRUS LABORATORY CASE DEFINITION**

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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 are Murray Valley encephalitis virus (MVEV), dengue virus (DENV) and the Kunjin strain of West Nile virus (KUNV). MVEV is closely related to Japanese encephalitis virus (JEV), which has caused a small number of human infections in the Torres Strait Islands and Cape York. Other flaviviruses uncommonly causing human infection within Australia are Kokobera (KOKV), Alfuy (ALFV), Stratford (STRV) and Edge Hill (EHV), though 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 maintainactivity during epidemics.

DENV is widespread in south Asia, sub-Saharan African, 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 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. A dengue vaccine developed by Sanofi Pasteur has been licensed in several countries after promising phase III trials[6]. There are several other dengue vaccines in development.

MVEV is the major cause of arboviral encephalitis acquired in Australia. It is endemic in the Kimberley region of 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*[7], the major vector for MVEV as well as KUNV and JEV[8]. 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[7]. Between 1975 and 2010, apart from several cases in central Australia in 2000 following heavy rainfall[9] 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 at 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.

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 the south-east of 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 recent review10 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. In 2011, there was an outbreak of KUNV among horses in western NSW, with almost 1,000 cases and a 9% mortality11. KUNV activity was also found in Queensland, Victoria and SA during this outbreak, though no human cases were reported in those states[12]. Molecular studies found this strain to have a mutation in the NS protein that may have contributed to the unusual severity of disease[13].

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 a polyarthralgic illness in a handful of patients.

JEV causes a similar illness to MVEV. It is endemic to southern and eastern Asia and is now found regularly in the Torres Strait Islands, which led to a single introduction into mainland Australia at Cape York, without establishing endemic activity. The last case of JEV encephalitis acquired on the Australian mainland was in 1998[8]. JEV is maintained primarily in a mosquito-pig cycle and a serological survey in domestic pigs found widespread JEV activity in north Queensland in 1998[14]. Cases of JEV acquired overseas are occasionally seen in Australia[8]. Several vaccines against JEV are available.

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[8]. In the 2011-2012 period, DENV was the second most commonly notified arboviral infection in Australia (after Ross River virus), with 1446 reported cases, only 18 of which were known to be locally acquired. Yellow fever (YFV) is not present in Australia but may be seen occasionally in people arriving from overseas or post-vaccination disease may occur.

Zika 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[15], 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%[16]. 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[17, 16]. Most recently the South American strains have 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*, *Aedes hensilii*, and *Aedes 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-Barré 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. While this has affected a very large number of pregnancies in the Americas and Asia, the exact risk to individual pregnancies is yet to be determined. Several cases of ZIKV infection acquired overseas have been identified in Australia since 2013[18]. 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 have a very restricted availability, and serology may misdiagnose these infections as being due to one of the more common flaviviruses, or IgM may be missed due to absence of a specific test. It is important that laboratories bear this in mind when one of these is suspected and refer the samples for specific tests if necessary. Problems may also be encountered when patients have had JEV or YFV vaccination, where antibody responses to any infecting flavivirus may be modified or post-vaccination responses may be misinterpreted as indicating recent infection. There is little data on IgM responses following vaccination. Though they are likely to be attenuated compared with natural infection, they should be considered as a cause of a misleading positive IgM response, particularly if vaccination was recent. YFV IgM has been detected up to 3-4 years post-vaccine[19].

2. Laboratory Diagnostic Tests

2.1 Culture

Usually virus can only be recovered within the first few days of illness prior to the appearance of antibody.

2.1.1 Cell culture

Where culture is attempted, the specimen is initially inoculated on to a mosquito cell line (C6/36 is most commonly used) and incubated for 3-4 days at 28^{o}C. In order to get a cytopathic effect (CPE) this must be blind passaged to mammalian cell lines such as Vero or BHK cells and incubated at 37^{o}C for a few days. Virus is then usually identified by the binding of specific monoclonal antibodies, by neutralisation with specific antisera, or by specific nucleic acid detection tests.

2.1.2 Animal inoculation

Inoculation of specimen into suckling mouse brain and intra-thoracic inoculation of mosquitoes have been used, but are now are rarely performed.

2.2 RNA detection by nucleic acid testing

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

Since the 1990s, numerous studies evaluating 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[20,21]. The main advantage of dengue RT-PCR is a 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[22], dengue 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 3, falling to 50% by day 5[23]. 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[24], though the duration is shorter and the viral load is lower than in primary infection, independent of development of severe dengue[25]. 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[25], though RT-PCR is more sensitive than antigen detection in secondary cases[48].

Many of the published RT-PCR assays are developed in-house, though a number of commercial assays are now available. A 2014 study evaluated four commercially available RT-PCR assays compared to an in-house hemi-nested RT-PCR[26]. 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.

There is very little experience with the use of NAT for detection of MVEV and KUNV in humans, though assays have been developed. MVE-RNA has been detected in serum[27] and CSF[28] of cases. However, sensitivity and specificity figures are not available and assays are currently limited to reference laboratories.

NAT assays for detection of other flaviviruses rarely found in Australia such as JEV, ZIKV and YFV have also been developed, and commercial assays are available for some. ZIKV PCR has proven useful in the confirmation of cases in returned travellers[29,18]. The virus 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 fetal 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.

2.3 Serological tests

2.3.1 Antigen detection tests

The only widely available flavivirus antigen detection test is for dengue NS1 antigen. Antigen detection tests for CHIKV have also been described[30]. Fluorescent antibodies and immunohistochemistry have been used to identify flavivirus antibodies in tissues but are not routinely used in acute diagnosis[5].

2.3.1.1Dengue NS1 antigen test

The first enzyme immunoassay to detect the dengue non-structural protein 1 antigen (NS1) in serum was developed in 2000. The test has now become widespread for use in diagnosis of DENV[31], 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. NS1 levels then decline. Detection rate is inversely proportional to the presence of IgG antibodies though NS1 has been detected up to day 14 after onset of illness[32].

As with viraemia, the duration of antigenaemia is significantly shorter in secondary dengue than in primary[25]. 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 sensitivity of NS1 Ag for diagnosis of secondary dengue is therefore lower than for primary dengue[32].

Controls

Kit controls as specified for commercial kits. A low positive patient serum is also recommended as an external control for batch-to-batch variation.

Criteria

Sample optical density above the predetermined cut-off value. Controls within the recommended range for commercial kits. External negative control and positive controls show appropriate reactivity.

Sensitivity

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].

Specificity

Dengue NS1 antigen appears to be highly specific for DENV though cross-reactivity studies are limited[33]. False-positives have been reported. For example, a Vietnamese study published in 2009 tested 459 patients with acute fever[34]. Two patients were reported as having false-positive NS1 Ag EIA tests, one of which was strongly positive.

2.3.2 Antibody detection tests

These are the only tests used routinely outside reference laboratories for the diagnosis of flavivirus infections other than dengue. The flaviviruses evoke antibodies that are widely cross-reactive within the genus. This causes considerable difficulty in characterising antibody to the level required to identify the infecting virus. Furthermore, due to the 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[27].

For classical dengue, laboratory characterisation of the antibody is usually unnecessary due to the confidence of clinical diagnosis[35]. However for other illnesses that are either clinically difficult to differentiate and/or of major public health significance, full characterisation of the antibodies is desirable.

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 kit insert should be consulted. Acute samples may be collected at any time after the onset of illness. Convalescent samples should be collected 2-4 weeks after onset of illness. Occasional patients may take over 4 weeks to develop antibody.

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. Detection of flavivirus IgG may indicate recent or past infection with any flavivirus, or may be due to flavivirus vaccination (against YFV or JEV). It is of little value in diagnosis of recent infection unless paired sera are tested. 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. If an EIA is to be used for titrating antibody, then doubling dilutions should be used with loss of reactivity as an endpoint. For IgM tests that do not use an IgM capture format, it is essential that rheumatoid factor and IgG be removed from the sample before testing to avoid false positives and false negatives.

Controls

Kit controls as specified for commercial kits. A low positive patient serum is also recommended as an external control for batch-to-batch variation. For in-house tests, known positive (preferably high and low) and negative samples should be included. For IgM tests the control serum should come from a case of infection due to that virus. However, it is recognised that suitable samples may not be available for the less common infections. In those situations a serum with IgM to another flavivirus may be used provided it shows reliable and consistent cross reactivity. For IgG tests, serum with antibody to any flavivirus may be used as a positive control.

Criteria

Sample optical density above the predetermined cut-off value. Controls within the recommended range for commercial kits. External negative control and positive controls show appropriate reactivity.

Sensitivity

EIAs are thought to be highly sensitive for the detection of flavivirus IgG, although there has been considerable variation in the evaluations[36]. Based on the experience with indirect IFA for MVE, IgG antibody may be absent in early infection. Detection of IgM was found to be 84-88% sensitive in acute JE using an IgM capture EIA[36,37,38], and a dot EIA was 73% sensitive for admission samples[39]. Detail of duration after onset was not given. Han *et al*[39] found IgM in serum in 75% of cases within 4 days of onset. Sensitivities of 90-100% are reported for the dengue IgM EIAs[40,41,42]. The sensitivities of IgM assays for other flaviviruses have not been established. IgM is detected in the CSF in only about 70% of acute JE cases, peaking about a week after presentation[43, 44, 45]. CSF IgM has also been detected by EIA in a similar percentage of MVEV encephalitis cases and in rare cases of dengue encephalitis[46].

Specificity

IgG-EIA appears to be specific for flavivirus antibody, with high degree of agreement between different assays[36]. However, it will not differentiate between IgG to different flaviviruses. The IgM assays have generally had limited evaluation for a range of flaviviruses and should not be assumed to be specific. In evaluations of dengue and JE IgM kits, they varied in how often they detected IgM to the other non-infecting flavivirus[39]. Specificity of IgG can be achieved by using an epitope-blocking approach, where monoclonal antibodies to species-specific epitopes are used to inhibit the binding of patient serum[47]. If inhibition occurs then there is a significant amount of specific antibody in the patient's serum. A similar principal is involved in a competitive EIA, and an apparent JE-specific EIA has been recently reported[48]. The epitope-blocking and competitive EIAs detect total antibody (IgG and IgM) and are not, in themselves useful for proving presence of IgM. Also specific blocking may not be demonstrable early after onset due to the lower specificity of IgM and early IgG responses. Testing of convalescent serum is recommended for determining species specificity of flavivirus IgG.

2.3.2.3 Immunofluorescent Antibody (IFA)

Many are only available as in-house tests in reference-level laboratories, and use an indirect IFA format. Tests 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 such as Vero cells. 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.

Controls

Infected and uninfected cells should be used. This can be done on a single cell spot if the level of infection of the cells is sufficient to provide a satisfactory mix of infected and uninfected cells. Known positive (preferably high and low) and negative samples should be included. If a weak positive control is not used, then the reactivity for the positive controls should be semi-quantified and be as strong as usual. It is recommended that two independent persons read slides. For IgM tests the control serum should come from a case of infection due to that virus. However, it is recognised that suitable samples may not be available for the less common infections. In those situations a serum with IgM to another flavivirus may be used provided it shows reliable and consistent cross reactivity. For IgG tests, serum with antibody to any flavivirus may be used.

Criteria

Uninfected cells do not show any fluorescence. Negative control and positive controls show appropriate reactivity. Results match for the two readers.

Sensitivity

Presumed to be highly sensitive and likely to be similar to EIA.

Specificity

IgG-EIA appears to be specific for flavivirus antibody, but it will not differentiate between IgG to different flaviviruses. The IgM assays have generally had limited evaluation for a range of flaviviruses and should not be assumed to be specific. 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 6 also had a KUNV IgM detected and in all cases the reactivity was weaker than the MVEV IgM. For 8 samples from KUNV cases with positive IgM, 1 had a weak MVEV IgM detected also. CSF IgM was specific for the infecting virus, but data is limited.

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. HI measures total antibody (IgG and IgM) but is less sensitive than EIA or IFA for detecting IgM. Most of the antibody measured by the HI is IgG, particularly in convalescent samples. HI can be used for detecting IgG or IgM alone, but this requires physical separation of the two classes of antibody, classically by sucrose density gradient centrifugation, prior to testing. Antigen is prepared from the supernatant of virus grown in a permissive cell line such as Vero cells. The IgM fraction can be confirmed by showing loss of activity in the IgM fraction following treatment with an IgM dispersant such as mercaptoethanol or dithiothreitol (DTT). Antigen can be prepared for any flavivirus that will grow in a suitable cell culture, so assays to new or uncommon viruses can be prepared and performed in-house.

Controls
Serum control (serum plus goose RBC to check for nonspecific haemagglutinins), RBC control (to check for auto-agglutination of the RBC), antigen control for each antigen (antigen plus goose RBC to ensure that haemagglutination is satisfactory), positive control serum of known titre, negative control serum.

Criteria
No evidence of nonspecific agglutination or auto-agglutination, antigen controls within expected range, positive control serum within expected range, negative control serum negative.

Sensitivity
HI titres are relatively insensitive for both IgG and IgM compared with the EIA and IFA tests, but are valuable for determining comparative antibody titres between acute and convalescent sera.

Specificity
HI antibody responses are usually highest to the antigen of the infecting virus, and may give some clues as to the likely cause. However, they are insufficiently specific to confirm the virus and, if it is a second flavivirus infection, they may be misleading. Due to the broad cross-reactivity, it is generally accepted that any flavivirus antigen can be used to test for antibody to the range of flaviviruses, though the sensitivity will be highest for antibody to the virus from which the antigen was derived.

2.3.2.5 Neutralisation Titres

Traditional neutralisation titres (NT) 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 are technically easier, but 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 tire or FRNT) and PCR-based methods have been developed for some. Any of these can be used provided that the appropriative range of viruses is tested, and proper criteria are used for interpretation (see below).

Controls
Known samples negative for flaviviruses. Positive control samples for all the flaviviruses being considered. It is recognised that positive control material may not be available for some of the less common viruses.

Criteria
Negative and positive controls show expected reactivity patterns. To confirm antibody to specific flavivirus, the titre of the test serum must be at least 1:40 to that virus and be at least four-fold higher to that flavivirus than to the others tested.

Sensitivity
Neutralisation titres are highly sensitive for low level flavivirus antibody, probably equivalent to EIA and IFA for IgG detection. They do not distinguish between IgG and IgM.

Specificity
Neutralisation titres are regarded as the "gold standard" for specificity and can generally be taken to indicate the infecting flavivirus. The exception is second infections where the early antibody response may be specific for the previously infecting flavivirus, though convalescent samples will usually show a dominance of antibody to the recent virus.

2.3.2.6 Other Serological Tests

Other tests are in the process of development for research or reference use, including improved monoclonal antibody epitope-blocking EIAs. These are potentially useful for improving the accuracy of serological diagnosis in humans, as well as more accurate animal sero-surveillance. For example, a number of commercial epitope-blocking EIAs for WNV have been described for use on human and non-human serum for surveillance and diagnostic purposes[49,50]. These assays are much simpler and quicker to perform than plaque reduction neutralisation assays, but current assays for flaviviruses commonly seen in Australia are all 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.

2.3.3 Sensitivity and Specificity of Serological Tests

Antibodies to the different flaviviruses generally cross-react widely, and the presence of IgM and/or IgG to one of these viruses is not sufficient, in itself, to indicate exposure to that virus rather than another flavivirus. The exception is classical clinical dengue in a person with a history of exposure in an area of known dengue activity. In that case detection of dengue IgM is sufficient to confirm dengue infection.

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 very 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, and therefore acute and convalescent sample should be obtained wherever possible to test for rising IgG. 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.

IgM usually persists for weeks or months after the initial infection. Therefore single samples with IgM should only be taken as presumptive evidence of recent infection, and further interpretation depends on the clinical and exposure history. Definitive serological diagnosis of recent infection requires demonstration of seroconversion from IgG negative to IgG positive, or a significant rise in IgG levels between acute and convalescent samples. In order to attribute the infection to a particular flavivirus, either the virus must be detected by culture or PCR, or specific antibody must be shown using specific serology such as neutralisation titres or an epitope-blocking EIA. Caution in interpretation is required where the person has previously been infected by another flavivirus, as they may initially show a rise in specific antibody to their previous flavivirus rather than the current one. Similar problems may arise if infection occurs in a person who has previously been vaccinated against JEV or YFV.

2.3.4 Serological response in primary and secondary dengue infection

The antibody response to DENV infection is affected by previous exposure to flaviviruses. In those with no previous exposure to any flavivirus by infection or vaccination, the primary antibody response is slow. IgM antibodies appear first, detectable in 50% by day 3-5 after symptom onset, increasing to 80% by day 5 and 99% by day 10. IgM then declines to undetectable levels by 2-3 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. See Figure 1.

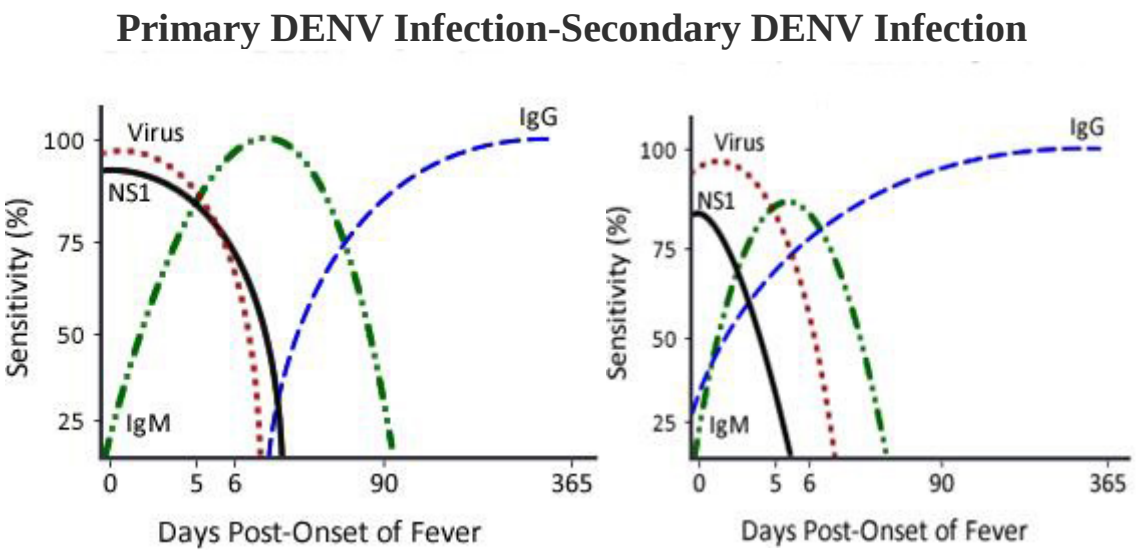

Figure 1. Taken from Centers for Disease Control and Prevention, Dengue Homepage>Laboratory Guidance and Diagnostic Testing (http://www.cdc.gov/dengue/clinicalLab/laboratory.html, retrieved 18/11/2016)

These differences in antibody responses have been used to differentiate primary and secondary dengue 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[51]. 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[52]. 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[53]. 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[52].

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[53]. 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 World Health Organization 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 7 days of illness) and there is a four-fold rise in IgG titre in serum collected during the convalescent phase.[54]

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 current value in the Australian population, but this is likely to changes as a larger numbers of Australian residents and visitors have had past flavivirus infection or vaccination.

2.3.5 Dengue rapid tests

A number of commercial rapid immunochromatographic tests for dengue are now available, targeting IgM and IgG or NS1 antigen or all three. Evaluations of older rapid tests for IgG/IgM detection reported low sensitivities. For example, a 2006 study reported only 2 of 8 of the evaluated rapid tests had a sensitivity >50%[55]. More recent evaluations of newer rapid tests that incorporate NS1 antigen detection show much higher sensitivities. A 2014 study evaluated four dengue NS1 rapid tests and found sensitivities of 71.9-79.1%, while the 3 evaluated EIAs had sensitivities of 85.6-95.9%[56]; all had good specificities of >95%. Another study, published in 2015, evaluated two dengue immunochromatographic tests for use within 14 days of symptom onset. One, testing IgM and IgG, had an overall sensitivity/specificity of 92.1%/62.2%. The other, testing IgM/IgG/NS1, had an overall sensitivity/specificity of 87.3%/86.8%[57].

False-positive NS1 Ag rapid tests have been described. For example, a case series from Singapore of two false-positive NS1 Ag tests using a rapid dengue IgM/IgG/NS1 kit in two patients with haematological malignancies was published in 2015[58]. The NS1 band in each case was weak. This is consistent with an earlier study of the same kit in 2012 that found two weak NS1 bands in patients with alternative diagnoses for their febrile illness[59].

2.4 Laboratory diagnosis of Zika virus infection

The virus can be detected in blood and saliva from the time of onset of illness for about a week. It persists in urine for another week[60] and has been found in semen up to 6 months after onset of illness[61]. Levels of RNA are higher and persist for longer in whole blood than serum/plasma, and possibly longer than in urine[62]. Viruses may also be found in placental and fetal 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 PCRs directed at different targets, including the NS5 and E genes. There are no commercial PCRs for ZIKV currently available in Australia.

The serological responses 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. The IgG responses are broadly cross-reactive with other flaviviruses and so antibody specificity must be proven by neutralization titres. To improve specificity, plaque or focus reduction assay with a >90% inhibition endpoint is recommended. The titre 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.

In patients who have been infected or vaccinated with another flavivirus the IgM responses are attenuated, and IgG rises very rapidly and may be primarily directed at the previously infecting virus rather than to ZIKV.

3 External Quality Assurance

RCPA Serology QAP for serology. 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[63].

4 PHLN Laboratory Definitions

4.1 Condition

4.1.1 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.

4.1.2 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.

4.1.3 Recent flavivirus infection

- **Definitive Criteria**
  Isolation of flavivirus from clinical material; OR
  Detection of viral 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.

- **Suggestive Criteria**
  Detection of IgM to a single flavivirus. 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. Where MVEV, KUNV or JEV infection is suspected, IgM tests should be done for antibody to DENV, MVEV, KUNV and JEV as a minimum. Where ZIKV infection is suspected, IgM tests should be done for ZIKV and DENV as a minimum. Where YFV infection is suspected, IgM tests should be done for YFV, KUNV, DENV, JEV and ZIKV as a minimum. 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.

4.2 Comments

The presence of IgM in a single sample must be interpreted in combination with the clinical illness and the likely exposure history to determine whether it may be recent infection, past infection, or a false positive IgM. Wherever possible, acute and convalescent samples should be collected to confirm recent infection. The antibody type should be confirmed by a specific serological test unless the virus itself has been detected.

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