Laboratory testing for Zika virus and dengue virus infections

Interim guidance

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World Health Organization

Executive summary

Zika virus (ZIKV) and dengue virus (DENV) remain significant public health threats. ZIKV infection is a cause of microcephaly and other congenital malformations and can cause neurological disease in children and adults. Persons infected with DENV are at risk of severe disease and death if not managed appropriately. ZIKV and DENV infections cannot be readily distinguished clinically; infections need to be differentiated from each other, and from other circulating arboviral and non-arboviral pathogens, using laboratory tests. This document provides revisions to the previous interim guidance published in 2016 on laboratory testing for ZIKV based on data and experience gathered during and after the Zika Public Health Emergency of International Concern (PHEIC) and incorporates current state of knowledge and guidance for DENV diagnosis [1,2]. In the absence of diagnostic randomized control trials to determine outcomes of comparative testing strategies, recommendations are based on panel review of the performance data of assays and the experience and observations made within their institutions through extensive arbovirus testing.

Updated key considerations, recommendations and good practices include:

- **ZIKV and DENV infections need to be differentiated from each other, and from other circulating arboviral and non-arboviral pathogens, using laboratory tests.**
- **Laboratory tests performed and interpretation of results must be guided by the interval between symptom onset or exposure, and the collection of specimens.**
- **WHO recommends the use of whole blood, serum, or plasma routine diagnostic testing for arboviruses, and urine for ZIKV NAAT testing.**
- **Molecular assays are the preferred detection method but the period of RNA detectability following infection is limited.**
- **Interpretation of serologic test results remains challenging because of cross-reactivity and prolonged detection of virus-specific antibodies; their utility depends on the patient's current and prior flavivirus exposures.**
- **Testing for antibodies to ZIKV and DENV should thus be done with careful consideration of epidemiologic and clinical context.**
- **For pregnant women, the diagnosis of ZIKV should always be based on laboratory evidence and testing in these patients should not be limited to a subset of samples, even during outbreaks.**
- **For pregnant women, accurate diagnosis is of particular importance; prolonged detection of RNA in blood and urine may facilitate. confirmation of ZIKV infection in these patients**
- **ZIKV IgM testing in pregnant women should be used with caution, since a positive test might reflect infection that occurred prior to pregnancy**
- **ZIKV testing for asymptomatic pregnant women remains challenging because of unknown optimal timing of specimen collection and risks of false positive and false negative results.**
- **Only laboratory tests that have undergone independent, comprehensive assessment of quality, safety and performance should be used for diagnosing arboviral infections.**
- **Any testing for the presence of ZIKV, DENV, and other pathogens in the differential diagnosis should be performed in appropriately equipped laboratories by staff trained in the relevant technical and safety procedures**

1. Introduction

1.1 Background

Zika (ZIKV) and dengue (DENV) viruses are arthropod-borne viruses (arboviruses) that are primarily transmitted by *Aedes (Stegomyia)* mosquito vectors and are related to other flaviviruses including yellow fever, Japanese encephalitis and West Nile viruses [3,4]. Sharing common mosquito vectors and ecology, ZIKV and DENV often circulate in the same geographic region. Infections with these viruses are frequently asymptomatic. Symptomatic infections are characterized by non-specific clinical features including fever, rash, malaise, headache, conjunctivitis, myalgia, and arthralgia. ZIKV infection tends to be mild and self-limited but rarely can cause more severe disease manifestations including Guillain-Barré syndrome in adults and children. ZIKV infection in pregnant women can cause congenital infection in their offspring and, in some cases, congenital Zika syndrome (CZS), which includes microcephaly and other congenital malformations; infection may also result in intrauterine foetal death. Persons infected with DENV are at risk of severe disease and death; secondary infections by other serotypes increase the risk of developing severe dengue. However, evidence-based management of suspected DENV infections has dramatically reduced mortality to <1% [5]. Of note, the clinical manifestations of acute febrile illness and/or rash seen in ZIKV and DENV disease are common to many infectious and non-infectious aetiologies that need to be considered in the differential diagnosis. In countries endemic for malaria, for example, it may be advisable to initially test for malaria parasites before testing for arboviruses depending on which pathogen is more prevalent at that time [6]

ZIKV outbreak activity was first documented in the Yap Main Islands, Federated States of Micronesia, in 2007, followed by reports of cases in Asia in 2010 [3]. However, the public health impact of ZIKV infection was appreciated anew during the rapid multi-country spread in the Western Pacific from 2013-15 and then in the Americas from 2015, when concerning ZIKV-associated congenital neurodevelopmental disorders associated with maternal infection during pregnancy were first recognized, as were increased rates of Guillain-Barré syndrome. In addition, sexual, congenital and perinatal routes of transmission were identified. Although epidemic ZIKV transmission has decreased substantially in the Americas, low-level endemic and sporadic transmission continues to occur in some countries within the region and in other parts of the world. In 2019, three autochthonous ZIKV cases occurred in France [7]. By contrast, DENV outbreak activity continues to increase, with record numbers of cases reported in Asia and Latin America in 2019, including areas previously impacted by ZIKV [8]. Laboratory testing is critical for accurate diagnosis, clinical management, and epidemiologic surveillance, including detection, tracking, and control of outbreaks.

Public health and clinical practitioners should be aware of locally prevalent pathogens, both to develop appropriate differential diagnoses and to identify circulating and emerging flavivirus infections or vaccine programmes that may confound serologic diagnosis, for example, Japanese encephalitis in Asia, yellow fever in Africa and South America, West Nile virus in North America and Europe, and tick-borne encephalitis in Asia and Europe [9].

This document provides revisions to the previous interim guidance published in 2016 [1] on laboratory testing for ZIKV and is based on data and experience gathered during and after the PHEIC for ZIKV infection and includes the following updates:

- more information is provided on selection and interpretation of laboratory diagnostic tests for both ZIKV and DENV as these viruses typically circulate in the same regions, and cannot be readily distinguished clinically,
- reiteration that ZIKV and DENV infections need to be differentiated not only from each other but also from other locally relevant circulating arboviral and non-arboviral pathogens, using laboratory tests;
- clarification on selection of specimen types based on data published after the 2016 interim guidance was posted;
- different testing strategies outlined for pregnant versus non-pregnant patients;
- broadened intervals between symptom onset and specimen collection to be considered for molecular testing for ZIKV in pregnant women where RNA detection provides more robust and specific evidence of recent infection;
- concession that if specimens collected within 7 days are negative by NAAT, IgM testing can be considered
- better delineation of the role and limitations of IgM and IgG antibodies for diagnostic use;
- the previous interim guidance encouraged collection of various specimen types for examination of ZIKV and neurological complications, microcephaly, and sexual transmission; however, those outcomes and modes of transmission have now been confirmed and thus specimen collection should focus on targeted diagnostic testing according to established protocols;
- description of the limitations of testing of asymptomatic pregnant women in the absence of data on optimal timing of testing and assays to be used
- removal of text pertaining to the emergency use assessment and listing (EUAL) as the WHO Prequalification Team ceased to assess new applications following the end of the PHEIC for Zika virus infection.

This interim guidance was developed by a panel of subject matter experts in *Aedes*-borne arboviral epidemiology and laboratory science. With waning ZIKV transmission, clinical evaluation of ZIKV and DENV diagnostic testing has become more challenging due to lower patient numbers. In the absence of diagnostic randomized control trials to determine outcomes of comparative testing strategies, recommendations are based on panel review of the performance data of assays and the experience and observations made within their institutions through extensive arbovirus testing.

1.2 Target audience

This interim guidance is for use by staff of laboratories testing for ZIKV and DENV infections and for clinical practitioners and public health professionals providing clinical management or conducting arbovirus surveillance. The guidance will require further adaptation at regional, national, and local level depending on epidemiology, healthcare priorities, resource availability, and capacity for diagnostic testing. The local epidemiology of ZIKV and DENV should be considered when applying and adapting this interim guidance (e.g., while differential testing is appropriate to determine a causative pathogen in an outbreak, during a documented DENV outbreak in the absence of ZIKV circulation, there is minimal role for ZIKV testing).

2. Interim recommendations

2.1 Viral dynamics

In ZIKV and DENV disease cases, symptoms typically appear a few days following infection but can occur up to two weeks later. Viremia for both viral infections is often detectable in the first few days of illness and wanes 5-7 days after symptom onset. Ribonucleic acid (RNA) levels during viremia are typically higher in DENV infections than in ZIKV infections [10]. Immunoglobulin M (IgM) antibodies typically increase to detectable concentrations by 7 days after symptom onset and can decline two to three months after infection. However, IgM antibodies have been detected for months to more than a year following infection, particularly with ZIKV [11]. Immunoglobulin G (IgG) levels rise soon after IgM and typically persist for years. In secondary DENV infections, IgG antibody levels are typically elevated more than IgM antibodies, which may be present at very low or undetectable levels/concentrations [12]. In addition, persons with prior flavivirus infections or vaccinations who are subsequently infected with ZIKV/DENV have been shown to develop an anamnestic response with a brisk quantitative increase in antibody titres against the earlier virus or vaccine; this response is referred to as “original antigenic sin” [13]. Viral dynamics are less clearly described in asymptomatic individuals and available data are limited to follow up of asymptomatic blood donors; however, concentrations of RNA and antibody classes appear to follow the same trajectory as symptomatic cases [14].

2.2 Laboratory tests

Laboratory tests for detecting infection with ZIKV and DENV include direct and indirect detection methods. Direct methods to detect viral material have high specificity and provide reliable evidence of current or very recent infection, whereas serologic test results are heavily influenced by population immune responses to other circulating flaviviruses, and due to longevity of antibody detection, are less helpful in determining how recently infection occurred. For any assay, the frequency of false positive results will increase in settings with low prevalence of infection.

Given the consequences of misdiagnosis, WHO strongly recommends that only laboratory tests that have undergone independent, comprehensive assessment of quality, safety and performance be used for diagnosing arboviral infections. These assays should only be used for their diagnostic indication on approved specimen types, collected within the appropriate timeframe, and in acceptable condition (see **section 2.4**). Furthermore, testing laboratories should participate in recognized external quality assessment programmes, where available, for all assays performed as part of the diagnosis of these arboviral infections.

Point of care tests for arboviruses have potential for use in many clinical settings; however, test performance is variable, and sensitivity and specificity of commercially available tests need to be systematically evaluated before they can be recommended for routine use [15]. In addition, they are subject to the same limitations as other non-point of care tests.

The testing strategy adopted by laboratories should be determined by the epidemiology of ZIKV, DENV, and other pathogens within the geographic region. The strategy should also take into account available resources and workflow of the laboratory. Testing approaches will vary depending on the prevalence of viruses known to be circulating in the area where the patients were exposed.

2.2.1 Detection of viral material

- **Nucleic acid amplification tests (NAAT)** are used for detection of virus-specific molecular targets on the viral genome and assays include reverse transcription-polymerase chain reaction (RT-PCR) and transcription mediated amplification. NAAT is the preferred method of diagnosis because of high virus-specificity and capacity to confirm infection; however, viral RNA is less likely to be detectable after the first week following symptom onset because of the short

duration and low amplitude of viremia [16]. Assays may also fail to detect RNA due to degradation if samples have not been properly stored or handled. When performed in accordance with rigorous protocols to avoid cross-contamination with amplification products and using validated primers and probes, false positive NAAT results are uncommon, emphasizing the need for external quality assurance and maintenance of proficiency [17].

- **Antigen detection tests** for use on blood specimens are currently only available for the detection of DENV infections and are targeted at the non-structural protein 1 (NS1) antigen, which is detectable over roughly the same period as viral RNA [18]. Tests include enzyme-linked immunosorbent assays (ELISA) and rapid point-of-care tests. Antigen detection immunohistochemical assays for use in tissue testing are available for ZIKV and DENV.
- **Virus isolation** can be performed in mammalian or mosquito cell culture for arbovirus molecular characterization like genotyping and lineage identification. It is more labour intensive and is slower to generate results, therefore it is rarely used for diagnosing infections. However, it remains the gold standard for direct identification and the capacity should be maintained in arbovirus reference laboratories for studies of pathogenesis, virulence, epidemic potential, or other studies of viral characterization.

2.2.2 Detection of immune response to viral infection

Testing for antibodies to ZIKV and DENV should be done with careful consideration of epidemiologic and clinical context [19]. Interpretation of results is complex because of cross reactivity with cocirculating viruses and prior infection with flaviviruses or history of flavivirus vaccination that can result in an anamnestic antibody response [13]. Furthermore, serologic tests have been shown to yield false positive results in patients with autoimmune conditions such as systemic lupus erythematosus. Serologic tests may be qualitative, quantitative, or semi-quantitative; only quantitative assay results should be used to interpret titre changes.

- **Serologic tests for detection of IgM antibodies** against ZIKV and DENV are commonly used to detect infections that have occurred more recently. IgM can be detected for months to over a year after infection, making it difficult to determine when infection occurred. Therefore, ZIKV IgM testing in pregnant women should be used with caution, since a positive test might reflect infection that occurred prior to pregnancy [11,14]. Available assays include enzyme immunoassays (EIAs), immunofluorescence assays (IFA), and for dengue, rapid point-of-care tests in various formats that detect IgM antibodies. Serologic testing should include all known circulating flaviviruses and those flaviviruses included in vaccination programs [20,21]. If both ZIKV and DENV are circulating in the region where a patient was exposed, detection of anti-ZIKV or anti-DENV IgM antibodies in the absence of IgM antibodies to the other virus (and absence of IgM against other circulating flaviviruses, if applicable) is considered presumptive evidence of recent infection with that virus but is not confirmatory. If testing indicates detection of both anti-ZIKV IgM and anti-DENV IgM antibodies, this is considered presumptive flavivirus infection in which the specific infecting virus cannot be identified.

However, if there is evidence that an outbreak in a localized area is due to only one of ZIKV or DENV, and this has been confirmed with a direct detection method, testing only for IgM against that virus might be more appropriate to define the disease burden and epidemiologic trends. For example, in a current dengue virus outbreak after cessation of ZIKV transmission, ZIKV antibodies might increase initially in previously infected persons, more so than DENV antibodies and create a false picture of disease aetiology.

- **Serologic tests for detection of IgG antibodies** in a single sample cannot be used for diagnosing acute infection since a single virus-specific IgG positive result is indicative only of prior infection with that virus, or a closely related virus against which cross-reactive antibodies may be present. Testing of paired specimens, the first collected typically within the first five days after symptom onset (acute specimen) and the second 2-3 weeks later (convalescent specimen), may be informative, particularly in persons who have not been previously infected with a flavivirus [5]. If seroconversion from negative to positive is demonstrated between the acute and convalescent specimens, this supports the diagnosis of an acute flavivirus infection; although this has been used in DENV diagnostics, IgG seroconversion is not a recommended method for diagnosing ZIKV infections as it has not been validated. If IgG antibodies are detected in both specimens and upon quantitative testing a ≥4-fold increase in antibody titre is observed, it may indicate secondary infection, but it will not be possible to determine whether ZIKV or DENV caused the most recent infection because of the aforementioned original antigenic sin phenomenon. IgG titre determination may be of prognostic use in management of secondary dengue infections, but clinicians should not wait for results before initiating dengue management protocols [2]. Available IgG assays include enzyme immunoassays (EIAs) and immunofluorescence assays (IFAs).

- **Neutralization assays** such as the plaque-reduction neutralization test (PRNT) are considered the serological gold standard for arboviral diagnosis and typically provide the greatest serologic specificity among available assay types; however, they are also subject to cross-reactivity in patients with prior flavivirus infection or immunization history [22]. In addition, PRNT is costly, requires highly trained staff, generates results that are complex to interpret, and should only be performed in laboratories with suitable containment facilities. For laboratories performing PRNT, a four-fold rise in neutralizing antibody titres to either ZIKV or DENV between the acute and convalescent specimens in the absence of antibodies to the other (and to related circulating flaviviruses) is confirmatory evidence of recent infection.

2.3 Case definitions and laboratory confirmatory criteria

Case definitions are available for ZIKV and DENV disease [5,23]; however, the specificity of the serologic confirmatory testing is markedly reduced in areas with prior flavivirus transmission. Therefore, regional, national, or local adaptations to the laboratory criteria for confirmed vs probable cases should be based on the current and prior epidemiology of ZIKV, DENV, and other flavivirus transmission within that jurisdiction. For example, in areas without prior flavivirus transmission, and with circulation of only ZIKV or only DENV, serologic criteria may be sufficient to meet confirmatory or probable classification because of low likelihood of cross-reactivity.

Patients who meet the suspect clinical case definition criteria and have a compatible epidemiologic exposure history, will be classified as:

- **confirmed cases** if they have evidence of ZIKV or DENV infection by direct detection of viral RNA in acceptable clinical specimens by NAAT (e.g., RT-PCR), viral isolation by culture, or for DENV, detection of DENV NS1 antigen by a validated immunoassay.

For persons with a low likelihood of prior flavivirus infection and no history of flavivirus vaccination, the following serological criteria may also be considered confirmatory: (1) positive anti-ZIKV or anti-dengue IgM antibodies in blood or CSF and PRNT titres ≥ 20 for that specific virus that is at least fourfold as high as titres for other flaviviruses, or (2) a four-fold rise in quantitative antibody titre for the specific virus in paired sera collected 2-3 weeks apart, i.e., during the acute and convalescent phases of infection and absence of antibodies to other flaviviruses endemic to the area of exposure.

- **probable cases** if they have detectable IgM antibodies in serum/plasma to either one of ZIKV or DENV but not the other, and no IgM antibodies against other endemic flaviviruses

Local ZIKV and DENV epidemiology should also be considered. For example, during an outbreak where only one of the viruses has been identified in an area, and there is reasonable certainty that the other virus is not cocirculating, detection of IgM antibodies against the virus known to be circulating might be considered a probable case for surveillance purposes, even if IgM testing is not performed for the other pathogen.

2.4 Specimens

RNA from both ZIKV and DENV been detected in blood, urine, saliva, cerebrospinal fluid, (CSF) and tissues; ZIKV RNA has also been identified in amniotic fluid, semen, saliva, rectal swabs, and other bodily fluids, although few specimen types have been validated for clinical diagnosis using available assays. Viral RNA is typically detectable in serum for less than 5 days after symptom onset in ZIKV and DENV infections; however, prolonged detection of ZIKV RNA in other specimen types has been documented [24-26]. The concentration of RNA in ZIKV infections is lower than in DENV infections [7]. ZIKV RNA may be detected in urine up to 14 days after symptom onset and in whole blood, based on longitudinal blood donor studies, ZIKV RNA can be detected for up to 3 months, likely due to viral association with red blood cells [14]. Use of whole blood thus allows a longer potential period of detection than serum or urine, but only among an estimated two-thirds of cases and under optimal research circumstances [14].

Based on available evidence related to specimen stability and reproducibility of results, WHO recommends the use of whole blood, serum, or plasma routine diagnostic testing for arboviruses, and urine for ZIKV NAAT testing [27]. Testing of whole blood for ZIKV infection should be considered, if validated testing is available, for symptomatic pregnant women with high risk of exposure to ZIKV because the extended period of detectable RNA in whole blood may allow for confirmation of recent infection. It could also be considered for asymptomatic pregnant women in areas of ZIKV transmission, however, the optimal timing of testing and performance in this subpopulation has not been evaluated [28]. Thus far, the only reported study in asymptomatic women comparing whole blood, serum, and urine testing showed no benefit of whole blood testing to improve detection of infections [29]. More widespread evaluation of the utility in pregnancy is needed.

In persons with neuroinvasive disease (e.g., encephalitis, meningitis) and possible exposure, CSF should be collected and tested for evidence of ZIKV or DENV, in

addition to other known neuroinvasive aetiologies [30,31]. For congenital ZIKV infection and autoimmune mediated neurological disease (e.g., Guillain-Barré syndrome), collection for the express purpose of diagnosing ZIKV infection is not recommended; however, CSF obtained as part of the routine diagnostic workup can be tested for the presence of viral RNA and virus-specific IgM to assist with determining aetiology.

The following types of specimens are those best suited for testing with the most commonly available ZIKV and DENV diagnostic assays. However, the product insert for the specific assay used should be reviewed prior to testing to determine the specimen types for which the use of the assay has been validated and approved.

2.4.1 Specimens suitable for NAAT (e.g., RT-PCR) to detect viral RNA:

- Serum or plasma collected from non-pregnant patients <7 days of symptom onset, or ≤14 days of symptom onset for pregnant women with suspected DENV or ZIKV infection. Serum should be collected in a sterile, dry tube without anticoagulants; plasma should be collected in an EDTA tube.
- Urine collected in a dry, sterile container ≤14 days of symptom onset for ZIKV-suspected cases.
- Whole blood collected in an EDTA tube for pregnant women

2.4.2 Specimens suitable for dengue NS1 antigen diagnostic tests:

Serum, plasma, or whole blood collected from patients within 7 days of symptom onset, depending on which specimen types have been validated for use with available assays.

2.4.3 Specimens suitable for serologic testing to detect antibodies:

- Serum or plasma collected from patients within 3 months after symptom onset

2.5 Patient information

The following data should be obtained at the time of specimen collection [32]:

- **Demographic information,** including name, date of birth, sex, pregnancy status, place of residence, and date and time of specimen collection;
- **Symptoms**, including date of onset and duration of symptoms;
- **Non-vector mediated exposure** such as unprotected sex with a known case of ZIKV or someone who had travelled to an area of ZIKV transmission, blood transfusion, or organ transplantation, or laboratory exposure; or in the case of an infant, whether they were nursing/consuming breast milk;
- Comprehensive history of travel in the prior month, and for the duration of pregnancy in pregnant women, including dates, place, duration of visit; and travel history of any sexual partners
- **Vaccination history** against flaviviruses including yellow fever, Japanese encephalitis, tick-borne encephalitis, and dengue viruses.

2.6 Who should be tested

The following groups should be prioritized for specimen collection and testing:

- patients with severe disease manifestations of ZIKV (e.g., Guillain-Barre syndrome) or DENV (e.g., severe dengue, dengue with warning signs) infection (figure 1);
- symptomatic pregnant women living in areas with ongoing (epidemic or endemic) ZIKV transmission (figure 2); testing can be considered for asymptomatic pregnant women in areas with high levels of epidemic transmission, taking into account test limitations discussed below;
- symptomatic pregnant women with a history of travel to areas with ZIKV transmission or whose sexual partner had known ZIKV infection or lived in or travelled to an area with ongoing ZIKV transmission;
- pregnant/post-partum women from areas with ZIKV transmission whose foetuses or newborns have evidence of congenital anomalies compatible with CZS;
- neonates, foetal deaths, and stillbirths with microcephaly or suspected congenital Zika syndrome delivered in areas with ZIKV transmission or born to women with a travel history or other potential exposure to ZIKV during pregnancy;
- infants with mothers diagnosed with ZIKV infection during pregnancy; and
- initial cases of suspect autochthonous infection with DENV or ZIKV in areas without ongoing transmission but known to be at risk for introduction or re-emergence.

During an epidemic, especially in areas with widespread transmission, it may not be feasible or cost effective to test every suspected case. Hence the national protocol must stipulate the percentage of samples to be tested for confirmation and frequency of random sample checks to determine epidemiological trends and detect any change in serotypes or virus in circulation. For pregnant women, however, the diagnosis of ZIKV should always be based on laboratory evidence and testing in these patients should not be limited to a subset of samples, even during outbreaks.

2.7 Testing strategy to detect new transmission of ZIKV or DENV

In areas at risk for new introduction or re-emergence of ZIKV and DENV, testing may be incorporated into local algorithms together with co-circulating pathogens e.g., CHIKV, malaria, influenza, Middle East respiratory syndrome coronavirus (MERS-CoV), severe acute respiratory syndrome coronavirus SARS-CoV-2, and other novel coronaviruses, either sequentially or in parallel. Detection of ZIKV or DENV in an area where it has not yet been recognized, or where transmission was interrupted, requires thorough confirmatory laboratory testing to verify local transmission. WHO and partner organizations are in the process of conducting global and regional assessments to determine best practices to detect emergence and re-emergence of ZIKV, DENV and other arboviruses. Those assessments will inform comprehensive guidance on surveillance practices (e.g., sentinel sites, syndromic surveillance, molecular monitoring) and the accompanying laboratory testing algorithms.

2.8 Testing strategy once ZIKV/DENV transmission has been confirmed in a region

Testing for both ZIKV and DENV would generally not be employed where it has been determined that a single aetiologic agent is confirmed as the cause of an outbreak and there is no current circulation of the other virus. Coinfection with more than one arbovirus has been documented though generally is uncommon.

Once transmission of ZIKV and/or DENV has been confirmed in a region, WHO recommends the following strategies:

2.8.1 Non-pregnant persons (Fig 1)

Only symptomatic patients with indications for testing (see section 2.6) should be tested.

- **presenting < 7 days after symptom onset**

  NAAT should be conducted among patients presenting <7 days of symptom onset. NAAT for ZIKV, DENV and other likely pathogens in the differential diagnosis, e.g., CHIKV, may be done simultaneously or sequentially taking into consideration evidence of co-circulation of these pathogens. In addition, or if only DENV is suspected, dengue NS1 antigen testing may be performed in addition to, or as an alternative to DENV NAAT. If NAAT and NS1 are negative, serum may be tested for IgM antibodies. If CSF has been collected, it may be evaluated to for the presence of RNA and if none is detected, for IgM antibodies.

- **presenting ≥7 days after illness onset**

  IgM serology should be performed on serum/plasma. A reactive result for IgM to ZIKV or DENV alone that is nonreactive to other flaviviruses endemic to the patient's geographic region of exposure suggests recent infection with that virus. If CSF has been collected, it may be evaluated to for the presence of IgM.

2.8.2 Pregnant women

While DENV infection in pregnancy poses risks of maternal death and obstetric complications such as haemorrhage and pregnancy loss, infection with ZIKV is of clinical concern because of the risk of congenital ZIKV infection and possible CZS, and foetal death. Though still under investigation, the proportion of foetal infections as a result of maternal ZIKV infection during pregnancy has been estimated to be 20-30%, and among those, the proportion who develop CZS ranges from 5-14% [3]. Research is also ongoing into the long-term sequelae of congenital ZIKV infection in children with or without CZS. Congenital ZIKV infections with characteristic developmental disorders have been noted whether pregnant women have reported symptoms or not. Prior to testing and upon receipt of laboratory results, it is important to ensure that the pregnant woman receives accurate information about the test(s) and limitations thereof. For test results indicating recent ZIKV infection, particularly IgM positive results in the absence of confirmatory evidence, the provider should communicate information to the pregnant woman on the risks of false positive results (particularly in low prevalence setting), as well as the prognosis of the pregnancy if the laboratory tests accurately reflect infection. Guidance for pregnancy management in the context of Zika virus infection is available through WHO at https://www.who.int/publications/i/item/WHO-ZIKV-MOC-16.2-Rev.1 and is being updated; the laboratory testing portion of that document is superseded by the information in this guideline [33].

If there is a high risk of ZIKV exposure, pregnant women should be tested as follows, depending on whether they show symptoms of ZIKV infection:

2.8.2.1 Symptomatic pregnant women (Fig 2)

- **Presenting <7 days after symptom onset**

  NAAT should be performed on blood (whole blood, serum, or plasma) AND urine specimens. If any specimens are positive, a repeat NAAT should be performed on newly extracted RNA from the same specimen to rule out false-positive test results. If NAAT fails to detect RNA on all these specimens, the woman is considered unlikely to be infected. However, since ZIKV and DENV RNA serum concentrations wane within the first week after

symptom onset, negative test results do not rule out infection. If NAAT is negative, serum may be tested for dengue NS1 if appropriate based on the epidemiology. If both NAAT and NS1 are negative, the serum may be tested for IgM antibodies. If ZIKV NAAT fails to detect RNA and no IgM antibodies are detected, infection is unlikely. If clinical concerns remain, a serum specimen collected ≥7 days following infection may be tested as outlined below.

- **Presenting 7-14 days after symptom onset**

  NAAT should be performed on blood (whole blood, serum, or plasma) and urine specimens since prolonged detection of RNA in serum and whole blood has been reported in pregnant women. If these results are negative, the serum should be tested for ZIKV IgM antibodies. If no IgM antibodies are detected, there is no laboratory evidence of ZIKV infection.

- **Presenting >14 days after symptom onset**

  IgM serology should be performed on serum/plasma. A reactive result for IgM to ZIKV or DENV alone that is nonreactive to other flaviviruses endemic to the patient's geographic region of exposure suggests recent infection with that virus. However, an IgM-positive test result might represent non-specific reactivity, cross-reactivity to a flavivirus that was not tested for, or residual IgM positivity from a previous flavivirus infection that occurred prior to the current pregnancy. Due to the particular need for a definitive diagnosis in pregnancy and the extended period of RNA detectability in this specimen type, additional NAAT testing of whole blood could be performed if a positive IgM result is obtained. If the NAAT does not detect RNA, infection is not ruled out and the IgM result remains presumptive. The limitations of the result should be conveyed to the provider for counselling and follow-up of the pregnant woman. In areas without high background flavivirus seroprevalence, PRNT (if available) may assist in identifying the infecting virus but will only be useful for assessing recency of infection if paired acute and convalescent specimens show ≥4-fold increase in antibody titre.

2.8.2.2 Asymptomatic pregnant women

During periods of epidemics or known risk of ZIKV exposure during pregnancy, strategies for testing of asymptomatic pregnant women remain challenging. Though universal screening is not recommended, laboratory testing can be considered but requires caution because testing of asymptomatic pregnant women creates multiple diagnostic challenges. NAAT has the highest specificity and thus accuracy; however, the limited duration of viremia and absence of a reference point of symptom onset makes detection of RNA unlikely when testing serum or urine of asymptomatic women. Testing of whole blood specimens for ZIKV infection can be considered for asymptomatic pregnant women with high risk of exposure to ZIKV, due to the extended period of RNA detectability in this specimen type but negative results do not rule out infection. Although serology can assist in identifying infections for a longer period, detection of anti-ZIKV IgM antibodies may represent non-specific reactivity, cross-reactivity to a flavivirus which was not tested for, or residual IgM positivity from a flavivirus infection that occurred prior to the current pregnancy.

2.8.3 Suspected congenital ZIKV infection

Testing for congenital ZIKV infection should be performed in clinical specimens collected from neonates with (1) mothers who tested positive for ZIKV infection during pregnancy, or (2) clinical features consistent with congenital Zika syndrome and potential maternal ZIKV exposure during pregnancy. Neonates should have serum and urine collected as soon as possible after birth for ZIKV testing by NAAT and for IgM testing. Cerebrospinal fluid (CSF), if collected as part of their clinical evaluation, should be tested for ZIKV by NAAT and for IgM. If serum or CSF from a neonate has detectable ZIKV IgM antibodies, additional confirmatory testing is warranted due to the potential for nonspecific reactivity of the assay. Because of the transfer of maternal IgG antibodies to the neonate, serum samples should be obtained at 18 months of age or older to verify the presence of virus-specific antibodies in the child, which would confirm the infection, assuming post-natal infection with ZIKV had not occurred. In neonates with these developmental anomalies, testing for other teratogenic infections should also be performed.

2.8.4 Suspected Zika-associated foetal death, stillbirth or second or third trimester spontaneous abortion

Maternal serum and urine should be collected for ZIKV testing by NAAT and serum for IgM testing. Foetal specimens (serum, tissue) should be tested by NAAT. It is not necessary to test these specimens for DENV infection.

2.9 Specimen processing and storage

Specimen processing and storage should follow standard operating procedures of the laboratory. Temperature should be monitored and recorded regularly to identify potential fluctuations. Domestic refrigerators/freezers with wide temperature fluctuations are not suitable for the storage of frozen specimens. Repeated freezing and thawing of specimens should be avoided.

To maintain nucleic acid integrity for NAAT or virus isolation, it is recommended that specimens be kept refrigerated at 2-8°C and tested within 48 hours. If there is a delay of more than 48 hours before testing, serum should be separated and stored separately. All types of specimens may be kept frozen at -20°C for up to 7 days. For storage longer than 7 days, specimens should be frozen at -70°C.

2.10 Biosafety

Diagnostic laboratory work, including reverse-transcription polymerase chain reaction (RT-PCR) analysis and serological testing on clinical specimens from patients who are suspected or confirmed to be infected with arbovirus infections, should be conducted under appropriate conditions including consideration to use a biological safety cabinet (BSC, class II) or other primary containment device for inactivation or extraction of the samples based on the risk assessment as described in the WHO Laboratory Biosafety Manual, 4th ed [34]. If virus isolation as well as testing for alphaviruses or other pathogens are performed, it will require particular caution, including further heightened control measures.

Any testing for the presence of ZIKV, DENV, and other pathogens in the differential diagnosis should be performed in appropriately equipped laboratories by staff trained in the relevant technical and safety procedures. National guidelines on laboratory biosafety should be followed in all circumstances, recognizing that specimens may harbour other, unsuspected high-risk pathogens.

2.11 Shipping specimens

Specimens known to be, or suspected of, containing ZIKV, or DENV may be shipped on dry ice (which requires UN Dangerous Goods shipping regulations), ice packs, or refrigerant packs as biological substances category B, UN3373. Coolant materials must be replenished during transportation to avoid thawing. If refrigerants, rather than dry ice, are used, they should preferably be processed within 24-72 hours of collection.

International regulations, as described in the WHO *Guidance on Regulations for the Transport of Infectious Substances 2021-2022* should be followed. [35]

2.12 Choosing laboratory tests (*in vitro* diagnostics)

Consideration must be given to the design and performance of the diagnostic products to ensure that testing is safe and effective, and that they have been evaluated for use in the geographic region where testing is to be performed. To date, only a limited number of commercially available ZIKV and DENV IVDs have undergone regulatory assessment of quality, safety or performance [36].

Ideally, a subset of specimens that test positive for RNA by NAAT should be forwarded to reference laboratories for further confirmation and sequencing, when available, to monitor for strain variations and maintain RNA reference material. Laboratories using a pan-flavivirus NAAT in combination with gene sequencing to detect ZIKV or using other conventional molecular methodologies such as multiplex assays, are requested to ensure in-house primer sequences have been updated to detect the recent ZIKV lineages. Primer and probe sets for various arbovirus-specific assays are available in the published literature.

Several institutions have developed in-house assays to test for arbovirus infections. WHO recommends that laboratories wishing to develop and perform in-house RT-PCR assays order the published primer/probe sets which are able to detect all circulating lineages of these viruses from their usual supplier and ensure that the assay is properly validated for use in each specimen type. Similarly, for commercial assays, laboratories should follow the manufacturer's instructions on specimen type, and if necessary, validate their assays for types of specimens and include appropriate process (internal) controls and external quality control. Quality control material is available from the global European virus archive (http://global.european-virus-archive.com/) and international standards for ZIKV antibodies and ZIKV RNA available through a WHO programme on international biological reference preparations (https://www.nibsc.org/products/brm_product_catalogue.aspx). WHO regional offices may be able to assist with this process.

3. Guidance development

3.1 Acknowledgements

The following individuals contributed to the development of this interim guidance:

External experts: Dr Ana Maria Bispo de Filippis, Instituto Oswaldo Cruz, Brazil; Dr Aaron Brault, United States Centers for Disease Control and Prevention, United States of America; Dr David William Graham Brown*, Public Health England, United Kingdom of Great Britain and Northern Ireland; Dr Amy Lambert*, United States Centers for Disease Control and Prevention, United States; Dr Marc Fischer, United States Centers for Disease Control and Prevention, United States; Dr Maria Guadalupe Guzman*, Instituto de Medicina Tropical Pedro Kourí, Cuba; Dr Erum Khan, Aga Khan University, Pakistan; Professor Marion Koopmans*, National Institute for Public Health and Environment, Netherlands; Maria Alejandra Morales, Centro Nacional de Referencia para diagnóstico de Dengue y otros arbovirus Argentina; Dr Jorge L. Muñoz, United States Centers for Disease Control and Prevention, United States; Dr Lee Ching Ng, National Environment Agency,

Singapore; Dr Bertrand Sudre, European Centre for Disease Prevention and Control, Sweden; Dr E Sreekumar, Rajiv Gandhi Centre for Biotechnology, India; Dr J Erin Staples, United States Centers for Disease Control and Prevention, United States.

The external expert group all submitted declaration of interest forms and no conflicts of interest were identified that would preclude participation in the guidance development.

WHO staff members and consultants from headquarters, regional and country offices: Dr Mathieu Bangert*; Dr Amal Barakat*; Dr Uzma Bashir Aamir; Dr Lauren Carrington; Dr May Chu; Dr Leticia Franco Narvaez; Dr Varja Grabovac; Dr Belinda Herring; Francis Inbanathan; Dr Eve Lackritz; Dr Jairo Andrés Méndez Rico; Dr Nora Monnier*; Dr Karen Nahapetyan; Dr Christopher Oxenford; Dr Dmitriy Pereyaslov*; Dr Ingrid Rabe; Dr Diana Rojas Alvarez; Dr Maria Van Kerkhove; Dr Raman Velayudhan; Dr Karin Von Eije.

WHO staff members and consultants are required to submit declaration of interest forms at the inception of each work contract and no conflicts of interest were identified that would preclude support of the guidance development.

*Participated in 2018 only

3.2 Guidance development methods

Experts in laboratory testing, arboviral epidemiology, and clinical virology with proven experience, prior publications, and expertise in arbovirus diagnostics. They were identified through existing networks of WHO Collaborating Centres, including experts from Africa, the Americas, Asia, and Europe. The expert group were initially convened at a meeting in Geneva on 11 October 2018 to review the draft guidance and were provided with a review package including the prior WHO interim recommendations, updates to partner agency guidance, and an agenda outlining key points identified in the literature review. All guidance documents published by regional offices and major public health agencies after 2016 were reviewed. There were a limited number of relevant publications as most were developed during the height of the pandemic and mainly agencies within the Americas continued to revise their guidance because of the ongoing concurrent circulation of ZIKV, DENV and CHIKV and guided by observations of laboratory testing practice and evaluation of results. Written feedback was provided by participants following the meeting and incorporated in the revised document. Based on the group discussion, a revised draft guidance document was developed by WHO staff, with some delay due to revisions in multiple agency guidances and notable publications identified during sequential PubMed database searches in 2019 that required further consideration. In 2020, after internal WHO review of the revised document, it was circulated again to the expert group, some of whom were heavily engaged in the COVID-19 pandemic response and who nominated colleagues with similar experience and credentials to participate in their stead. Finally, a virtual meeting was convened on 29 September, 2020, to finalize the current version of the interim guidance; literature identified during literature review conducted in 2020, was circulated along with the guidance. Literature review has continued on an ongoing basis since September 2020 to identify any developments that would require adaptation of the interim guidance in its current form.

Members of the expert group developed the recommendations based on expert opinion, interactive discussion on published data on assay performance, prospective data on molecular and serological analyte detection, and changes in practice advocated in other public health agency guidance. Good practice statements were included based on widely accepted laboratory and arbovirology principles including the need for use of adequately validated assays, selection of testing strategies based on prevailing epidemiology, differential testing for circulating related flaviviruses, and adherence to current biosafety manuals.

WHO continues to monitor the situation closely for any changes that may affect this interim guidance. Should any factors change, WHO will issue a further update. Otherwise, this interim guidance document will expire 2 years after the date of publication.

3.3 Declaration of interests

No competing interests were identified from the declarations of interest forms collected from all contributors. Funds from a United States Agency for International Development award to support the WHO Zika Virus Task Force were used to develop this interim guidance.

4. Research gaps

Identified gaps in the published data that are important for future updates to diagnostic testing guidance include (1) independent evaluations of diagnostic assay performance; (2) studies reporting performance of ZIKV testing in pregnant women across different geographic settings in different WHO regions where ZIKV transmission is known to occur; (3) randomized trials of diagnostic algorithms; (4) data on performance of testing in areas with infrequent ZIKV transmission and lower positive predictive value; (5) updated target product profiles for diagnostic assays for ZIKV and DENV; and (5) establishment of use cases for assays fit for purpose, for example, clinical diagnosis and management vs early outbreak detection.

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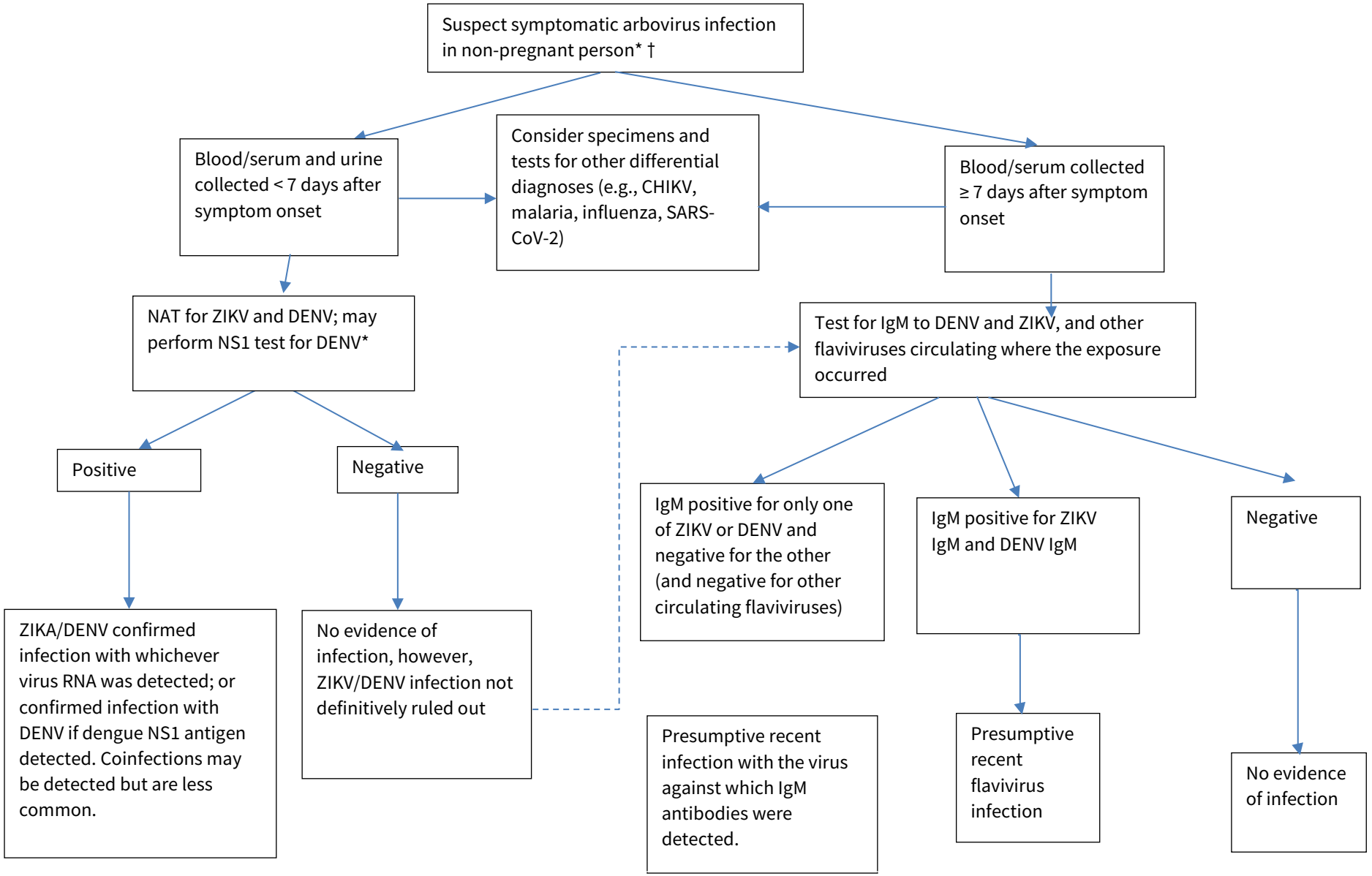
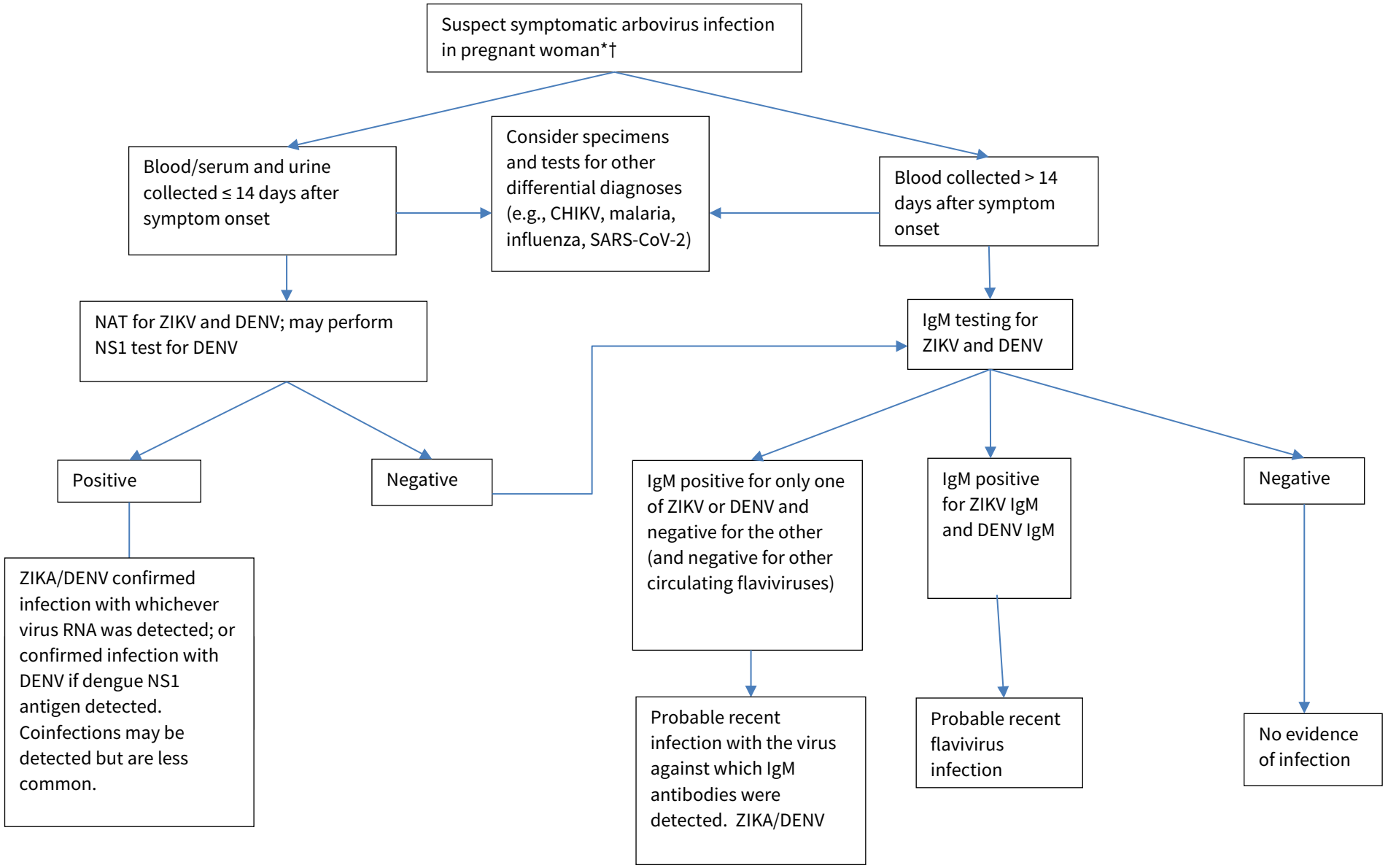
WHO reference number: WHO/ZIKV_DENV/LAB/2022.1



*Final interpretation of result should be done in conjunction with clinical presentation and epidemiological context
†Patient should be asked about prior flavivirus infections and vaccinations (e.g., yellow fever, dengue, tick-borne encephalitis, Japanese encephalitis)

Figure 1. Proposed testing algorithm for suspect Zika virus (ZIKV) or dengue virus (DENV) infection in a symptomatic non-pregnant person



*Final interpretation of result should be done in conjunction with clinical presentation and epidemiological context
†Patient should be asked about prior flavivirus infections and vaccinations (e.g., yellow fever, dengue, tick-borne encephalitis, Japanese encephalitis)

Figure 2. Proposed testing algorithm for suspect Zika virus (ZIKV) or dengue virus (DENV) infection infection in a symptomatic pregnant woman