Recommendations for laboratory detection and diagnosis of arbovirus infections in the Region of the Americas
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Abbreviations and acronyms

CDC  Centers for Disease Control and Prevention (United States of America)
cDNA  complementary deoxyribonucleic acid
CHIKV  chikungunya virus
CSF  cerebrospinal fluid
DENV  dengue virus
DNA  deoxyribonucleic acid
EEEV  eastern equine encephalitis virus
ELISA  enzyme-linked immunosorbent assay
FDA  Food and Drug Administration (United States of America)
GBS  Guillain-Barré syndrome
IFA  indirect immunofluorescence assay
IHC  immunohistochemistry
IPK  Instituto de Medicina Tropical Pedro Kourí (Cuba)
MAC-ELISA  IgM antibody capture enzyme-linked immunosorbent assay
MAYV  Mayaro virus
mRNA  messenger RNA
NS  nonstructural
OROV  Oropouche virus
PCR  polymerase chain reaction
PPE  personal protective equipment
PRNT  plaque reduction neutralization test
RELSA  Arbovirus Diagnosis Laboratory Network (Spanish acronym)
RNA  ribonucleic acid
RNase  ribonuclease
RT-PCR  reverse transcription polymerase chain reaction
SLEV  Saint Louis encephalitis virus
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>VEEV</td>
<td>Venezuelan equine encephalitis virus</td>
</tr>
<tr>
<td>WEEV</td>
<td>western equine encephalitis virus</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>YFV</td>
<td>yellow fever virus</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Zika virus</td>
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</table>
Introduction

Arthropod-borne viruses (arboviruses) are transmitted to humans primarily through the bite of hematophagous arthropod vectors (i.e., mosquitoes, ticks, sandflies, and biting midges). Over a 100 arboviruses are known to infect and cause disease in humans, with infection outcomes ranging from asymptomatic to life-threatening (1).

Arboviruses are a polyphyletic group of viruses that belong to several viral families and genera (1), i.e., Flavivirus (family Flaviridae), Alphavirus (family Togaviridae), Orthobunyavirus (family Peribunyaviridae), Phlebovirus (family Phenuiviridae), and Coltivirus (family Reoviridae). Most of these viruses have a single-stranded RNA genome (positive-sense for Flaviridae and Togaviridae and negative-sense for Peribunyaviridae and Phenuiviridae), while those of the Reoviridae family have a double-stranded RNA genome (1). The most relevant arboviruses in the Americas are flaviviruses, including dengue (DENV), Zika (ZIKV), yellow fever (YFV), West Nile (WNV), and Saint Louis encephalitis (SLEV) viruses, alphaviruses, which include chikungunya (CHIKV), Mayaro (MAYV), and equine encephalitis viruses, and the Oropouche virus (OROV), which belongs to the genus Orthobunyavirus.

This document was developed as part of the Regional Strategy for Arboviral Disease Prevention and Control, adopted by the Pan American Health Organization’s 55th Directing Council in September 2016 (2). It particularly responds to strategic line of action 4, which highlights the importance of strengthening the technical capacity of the Arbovirus Diagnosis Laboratory Network in the Region of the Americas or RELDA, by its Spanish acronym (2).

The purpose of this document is to provide public health laboratories in the Americas with technical recommendations on the samples and assays to be used for laboratory surveillance of arboviral diseases, and on the interpretation of laboratory results. The document will also be useful for research center and university laboratories that may need to identify arboviral infections as part of their research, to contribute to the characterization of arboviruses and to our understanding of their transmission dynamics.
Viruses included in this document were selected through expert consensus, and were grouped into three categories:

- Arboviruses that circulate or have recently circulated extensively in the Americas, and cause a significant burden, namely, DENV, CHIKV, ZIKV, and YFV.
- Arboviruses that have caused outbreaks in a limited number of countries and territories in the Region and have the potential for reemergence in the near future, namely, MAYV and OROV.
- Other neurotropic arboviruses that circulate in the Region, whose reemergence potential might be more limited but that can cause severe clinical conditions in humans and animals, such as equine encephalitis viruses, namely, eastern (EEEV), Venezuelan (VEEV), and western (WEEV) equine encephalitis viruses, as well as WNV and SLEV.

Description of the clinical presentation and management of these arboviral infections can be found in PAHO’s Tool for the Diagnosis and Care of Patients with Suspected Arboviral Diseases (3), which is mainly focused on diseases caused by DENV, CHIKV, and ZIKV but also reviews available information on diseases caused by WNV, YFV, OROV, MAYV, and equine encephalitis viruses. Differential diagnoses for these diseases have also been described elsewhere (3–5).
1.1 Arbovirus structure, genome, and life cycle
Viruses discussed in this document are enveloped and contain single-stranded RNA as their genetic material. This has several implications for viral detection: 1) enveloped viruses are usually less stable than naked viruses, which impacts virus isolation; 2) single-stranded RNA is chemically labile and easily degraded by ubiquitous ribonucleases (RNases). Thus, specific measures should be followed to maintain the integrity of the viral particle and its RNA genome during sample collection, handling, storage and transportation; 3) the genetic variability of RNA viruses impacts molecular diagnosis, as changes in the genome might affect the ability to detect the virus efficiently; and 4) within each family of arboviruses there are structural and genetic similarities and differences that may affect the ability to specifically detect these viruses.

1.1.1 Flavivirus structure, genome, and life cycle
The *Flavivirus* genus comprises more than 50 species, many of them transmitted by arthropods. Virions are spherical and enveloped, with a 40–60 nanometer diameter. The viral particle consists of an icosahedral nucleocapsid and a nonsegmented, linear, positive-sense single-stranded RNA genome of 10 to 11 kilobases (kb). The structure of the genome is generally conserved across members of the genus, with genes encoding three structural proteins: capsid (C), premembrane (prM) and envelope (E), as well as seven nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The virus life cycle begins with the attachment of the E protein to cell receptors and the internalization of the virus by endocytosis. The conditions inside the endosome promote the fusion of the virus envelope
with the endosomal membrane, and the capsid and the genomic RNA are released into the cytoplasm. The genomic RNA serves as a messenger RNA (mRNA) that is translated into a single polypeptide in the rough endoplasmic reticulum. The polypeptide is then processed into viral proteins by viral and host cell proteases. The viral genome replicates in the cytoplasm of the host cells through a negative-sense single-stranded RNA intermediate. New particles are then assembled, mature in intracellular compartments, and are released from cells by exocytosis (6).

1.1.2 Alphavirus structure, genome, and life cycle

Alphavirus is the only genus of the Togaviridae family. The alphavirus virion has a 70 nanometer diameter and contains a single copy of the nonsegmented positive-sense single-stranded genomic RNA of approximately 11 to 12 kb, an icosahedral nucleocapsid, and a lipid bilayer envelope displaying viral glycoproteins. The viral genome encodes a single polypeptide that is processed into five structural proteins: capsid (C); envelope proteins E2 and E3 (produced from a single precursor, pE2); 6K; and E1. The E2 protein recognizes host cell receptors, while E1 is involved in the fusion process. Nonstructural proteins (nsP1–4) are also produced from a single polypeptide, and participate in the replication of the viral genome, in the regulation of cell transcription and translation, as well as in the modulation of innate immune response. During infection, the virus binds to a host receptor and is endocytosed. The low pH of the endosome causes a structural rearrangement of the virion that induces membrane fusion and the subsequent release of the nucleocapsid into the cytoplasm. Viral RNA release from the nucleocapsid allows for the synthesis of nonstructural proteins, which activate both viral replication (through a negative single-stranded intermediate), and the transcription of an mRNA for the synthesis of structural proteins. In the cytoplasm, the new genomes are produced and assembled with C protein to form nucleocapsids, while the envelope proteins mature in the endoplasmic reticulum and the Golgi apparatus. Finally, the new viral particles are assembled and secreted from the cell membrane (7, 8).

1.1.3 Orthobunyavirus structure, genome, and life cycle

The Oropouche virus (OROV) is a member of the Orthobunyavirus genus, Peribunyaviridae family, the largest genus of RNA viruses, with more than 80 viruses. Orthobunyaviruses are enveloped and spherical, ranging in diameter from 80 to 120 nanometers. Their segmented genome is composed of three negative-sense single-stranded RNA molecules: L (large) of 6.9 kb; M (medium) of 4.5 kb; and S (small) of 1.5 kb. The S segment encodes the nucleocapsid and a nonstructural protein; the M segment encodes the precursor of envelope proteins Gc and Gn, and another nonstructural protein. The L segment encodes the RNA-dependent RNA polymerase (or RNA replicase). Binding of the virion to the host cells is mediated through the envelope glycoproteins Gc and Gn, and is followed by endocytosis, fusion and release of the nucleocapsid into the cytoplasm. The genome is transcribed, and the resulting mRNAs are translated to produce viral proteins. The genome is replicated through positive-sense RNA intermediates. The Gc and Gn proteins dimerize in the endoplasmic reticulum and pass to the Golgi apparatus where they are assembled with ribonucleoproteins (containing the viral genome, the nucleocapsid and the RNA replicase) to form new virions. Finally, viral particles are secreted from the infected cells (9).
Because of the segmented nature of its genome, OROV can recombine with other viruses of the Orthobunyavirus genus. This genetic reassortment generates diversity and may impact viral properties, for instance, pathogenicity. Several Oropouche reassortant viruses have been described in Brazil, Peru, and Venezuela, such as the Iquitos, Madre de Dios, and Perdões viruses (9, 10). These reassortant viruses have the S and L segments from OROV and unique M segments. Besides their biological relevance, reassortment may also impact the detection of these viruses.

1.2 Origin and evolutionary dynamics of arboviruses
Medically important arboviruses share a history of emergence from natural cycles originally involving sylvatic species of arthropods (mainly mosquitoes) as vectors, and vertebrates (mainly mammals and birds) as amplifying hosts (11). Arboviral emergence is a relatively frequent process, as exemplified by the independent emergence of DENV 1, 2, and 4, for which the existence of exclusive sylvatic ancestors has been demonstrated (12).

1.2.1 Main factors for arboviral emergence
Arbovirus emergence and epidemics can be considered ecological and evolutionary issues, as they mainly depend on the physical contact between humans and viruses in enzootic or epizootic cycles, and on the adaptation of the viruses to a new host and vectors from urban areas. Humans living in rural areas near the forest, the presence of bridge vectors feeding from both sylvatic vertebrates and humans, hunting, deforestation, mining, and every human activity on rural and sylvatic areas increase the risk for viral emergence from sylvatic sources (13, 14). Climate change, also a consequence of anthropogenic activity, has been linked to the expansion of vector distribution, and is, therefore, considered another important factor for viral emergence (15).

Most arboviruses are RNA viruses whose rapid evolution is the result of the convergence of high mutation rates, large population sizes, and short generation times (16). The various steps of the virus transmission process are a challenge for RNA viruses, which respond by the generation of genetically heterogeneous viral populations or quasispecies (17). Virus variants can become fixed, extinct or maintained as a polymorphism through natural selection (according to their selective advantage) and genetic drift (randomly) (18).

1.2.2 Viral emergence and transmission cycles
Arbovirus emergence in human populations has been previously described as a step-by-step process involving incidental infection through direct contact with natural reservoir hosts or vectors from the sylvatic cycle. Such an infection can progress through different stages (from sporadic to frequent), and establish exclusive human-to-human transmission by vectors in the urban cycle (19). There is evidence of previous and current sylvatic/enzootic cycles for arboviruses that circulate in urban settings, supporting their emergence/reemergence from sylvatic ancestors (20–23).

Arboviruses can be categorized in terms of their emergence and transmission cycle into three groups. The first group consists of viruses that circulate in an urban cycle causing epidemics, and are endemic...
in several countries, such as DENV, CHIKV, and more recently, ZIKV. YFV was also part of this category until the first half of the twentieth century, when vaccine development and Aedes aegypti mosquito vector eradication campaigns interrupted its urban cycle in the Americas (24). Arboviruses that have successfully established an urban cycle are maintained by different factors, the most important being an increase in human population density. In the first spillover from sylvatic cycles, arboviruses find an immunologically naïve human population. If there is a high-density competent vector population, transmission might be effective, and an epidemic behavior established. Several factors influence mosquito breeding in urban settings, including unplanned urbanization with deficient sanitary services and water availability, lack of awareness of the problem, and little active community involvement in vector control. These viruses can reach epidemic levels when susceptible humans allow for sustained transmission; subsequently, the incidence decreases as herd immunity increases. Virus persistence during interepidemic periods, when the number of susceptible humans or vector densities are extremely low or nonexistent (e.g., during the dry season), may be the result of vertical transmission in the mosquito vector, or due to egg tolerance to desiccation and survival for long periods (25, 26).

The second group comprises arboviruses with potential for or demonstrated human-to-human transmission, but currently circulating in an enzootic cycle that involves wild vertebrates and mosquito species. These viruses cause sporadic cases in humans by direct spillover from enzootic or bridge vectors, as the result of humans living or working in close contact with natural areas, in the so-called “emergence zone.” YFV is a current example, with a major outbreak in Brazil that began at the end of 2016 without evidence of human-to-human transmission, through urban vectors, in particular, the Aedes aegypti mosquito (27, 28). OROV enzootic circulation has been described in the Americas with sporadic human cases, as well as sustained transmission by an urban vector (e.g., Culicoides paraensis) (9, 10, 29). MAYV can also be classified in this group, as two recent cases of MAYV/DENV and MAYV/CHIKV coinfections in an area with no known reservoirs suggest that human-to-human transmission of MAYV might occur (30, 31).

Enzootic transmission of DENV, CHIKV and ZIKV in the Americas has not been observed. However, its potential establishment is of public health concern (32, 33), as evidence of infection or exposure in wild vertebrate species and mosquitoes has been accumulating (34–37).

The third group consists of arboviruses with established epizootic cycles involving wildlife or domestic animals as amplification hosts or reservoirs, and sporadically affecting humans as a dead-end host (11). The main feature limiting epidemic behavior among these viruses is the fact that humans do not display sufficient viremia to allow a successful second-round transmission (38, 39). However, their circulation in amplifying animal hosts, which are often in close contact with humans, is a significant threat, and should be a focus of epidemiological surveillance. WNV, VEEV, EEEV, WEEV, and SLEV, among others, belong in this group.
1.2.3 Genetic variability of arboviruses

RNA viruses are rapidly evolving biological entities. This property is mainly due to the lack of proofreading activity of the viral replicase or RNA-dependent RNA polymerase. Thus, mutations are incorporated at high rates, ranging from $10^{-4}$ to $10^{-6}$ substitutions/nucleotide/round of replication, which represent an average of one mutation per replicated genome for a typical 10 kb genome (40, 41). As up to $10^{12}$ viruses per day are produced in an individual host, there is an equivalent number of accumulated mutations, with the potential of escaping any adverse condition. Controlled experiments with viral clones of reduced variability have demonstrated that RNA viruses rapidly establish a heterogeneous intra-host population of mutants, favoring their adaptive potential, and, therefore, the colonization of new hosts (42). Viral populations are naturally selected according to the fitness of each variant and the chance of being transmitted to a new host (43). Therefore, the adaptation of the virus to two or more species necessary for its life cycle (human species and arthropod species) limits the accumulation of mutations and, to a certain extent, its evolution. In in vitro and in vivo studies, the risk of viral emergence and the role of different vector and host species have been assessed, and minimal nucleotide substitutions which modulate the ability of an arbovirus to replicate in a host have been described. Examples include the CHIKV A226V mutation as an adaptation to the Aedes albopictus mosquito during the reemergence of CHIKV in the Indian Ocean (44); changes from enzootic to epizootic/epidemic dynamics related to mutations in VEEV E2 gene (45, 46); and mutations linked to an increase of ZIKV transmission potential (47). The genetic variability of RNA viruses, however, is limited by the error threshold at which the variability is compatible with the maintenance of protein function and viral integrity. Also, arboviruses should be able to replicate in the vertebrate host and the phylogenetically distant vector, which poses a challenge for the virus.
Collection, conservation, and transportation of samples

2.1 Samples for the diagnosis of arboviral infections

The types of samples that can be used to diagnose arboviral infections depend on the suspected infection to be diagnosed, the assays to be used, the time since the onset of symptoms, and the accessibility of the sample. The main types of samples are described in Table 1.

In addition, the use of dried blood spots on filter paper for the detection of various arboviral infections by virological and serological methods has been described (48–53). To implement the use of these samples the following should be considered: the abundance of the analyte that is being detected (viral RNA or antigen, antibody) in capillary blood, the type of filter paper, and the conditions of transport and storage (54).

To guarantee the reliability of test results it is essential to comply with the criteria established for the collection, handling and shipment of samples. These criteria and standards must be elaborated by the local public health authorities and include the following fundamental points:

- Qualified and trained personnel in all the steps of the collection, handling and shipment of the samples.
- Protocols and quality standards established for sample collection and the collection of patient information.
• Hygiene and biosafety conditions in the sampling areas.
• Correct storage of samples and traceability of these and patient information under quality standards.
• All samples must be collected, stored and shipped in plastic containers (tubes and vials, among others) approved for clinical use and must be clearly labeled. Glass containers should never be used.
• Maintenance of the cold chain to guarantee the integrity of the samples. The cold chain is essential for molecular methods, which require undegraded viral RNA (and for viral isolation, for which the integrity of the viral particles must be maintained). When the cold chain is difficult to maintain, RNA stability in body fluids or tissues can be maintained by adding commercial RNA stabilizing solutions.
• Compliance with standards for the use and storage of patient data and personal information.

### TABLE 1  Samples for the diagnosis of arboviral infections

<table>
<thead>
<tr>
<th>SAMPLE TYPE</th>
<th>RECOMMENDED QUANTITY</th>
<th>MINIMUM QUANTITY</th>
<th>SAMPLE COLLECTION AND TRANSPORT</th>
<th>HANDLING AND TRANSPORT TEMPERATURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total blood without anticoagulant</td>
<td>5 ml</td>
<td>1 ml</td>
<td>Tube without anticoagulant</td>
<td>2–8 °C&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum</td>
<td>2.5 ml</td>
<td>0.5 ml</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Total blood with anticoagulant</td>
<td>5 ml</td>
<td>1 ml</td>
<td>Tube with EDTA or other anticoagulant&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2–8 °C</td>
</tr>
<tr>
<td>Plasma&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.5 ml</td>
<td>0.5 ml</td>
<td>No additives&lt;sup&gt;b&lt;/sup&gt;</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Urine</td>
<td>5 ml</td>
<td>1 ml</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Saliva</td>
<td>2.5 ml</td>
<td>0.5 ml</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Cerebrospinal fluid</td>
<td>1 ml</td>
<td>0.25 ml</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Amniotic fluid&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1 ml</td>
<td>0.25 ml</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Fresh tissue</td>
<td>1 × 1 × 1 cm</td>
<td>0.5 × 0.5 × 0.5 cm</td>
<td>No additives</td>
<td>≤ 2–8 °C</td>
</tr>
<tr>
<td>Tissue in RNA stabilizing solution</td>
<td>1 × 1 × 1 cm</td>
<td>0.5 × 0.5 × 0.5 cm</td>
<td>RNA stabilizing solution</td>
<td>According to manufacturer’s instructions</td>
</tr>
<tr>
<td>Fixed tissue in formalin</td>
<td>1 × 1 × 1 cm</td>
<td>0.5 × 0.5 × 0.5 cm</td>
<td>10% neutral buffered formalin&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Room temperature or 2–8 °C</td>
</tr>
<tr>
<td>Formalin-fixed paraffin-embedded blocks</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>Room temperature or 2–8 °C</td>
</tr>
</tbody>
</table>

Notes:

- <sup>a</sup> Do not freeze.
- <sup>b</sup> For molecular methods, avoid heparin as anticoagulant.
- <sup>c</sup> Plasma is considered equivalent to serum for most diagnostic testing, except for molecular testing in heparinized plasma. In this document, serum refers to serum or nonheparinized plasma, unless otherwise stated.
- <sup>d</sup> Only if collected for clinical reasons. Do not collect for the sole purpose of determining the etiologic agent.
- <sup>e</sup> Use approximately 10 times the sample volume.

EDTA: ethylenediaminetetraacetic acid; n/a: not applicable; RNA: ribonucleic acid.
2.2 Epidemiological and clinical information accompanying the samples

Samples should be accompanied by a minimal set of the patient’s epidemiological and clinical information to support the selection of the tests to be performed, as well as result interpretation. Such information is usually included in a standardized epidemiological form. Demographic information should include a personal identifier, age, sex, place of residence, pregnancy status (and gestational week), recent travel history (including destination and dates), and yellow fever vaccination history or other arboviruses (including dates). Clinical information should describe all signs and symptoms included in the arboviral disease case definitions and classifications (3, 4, 55), date of symptom onset, need for hospitalization (including need for intensive care), clinical diagnosis, and treating physician’s details. Relevant clinical laboratory results (complete blood count, tourniquet test results, urine analysis, liver enzymes) should also be provided, as well as information on sample collection (type, date, time, health care provider). Finally, the requested test(s) should be included. For fatal cases, the autopsy report should be included, if available.

2.3 Shipment of samples to the laboratory

General recommendations for sending samples to the laboratory are indicated below.

- Samples should be packaged according to local and international regulations (56). Triple packaging should always be used.
- Complete clinical and epidemiological records should be included.
- Samples should be shipped, if possible, within the first 48 hours of collection.
- If samples are shipped within 48 hours of collection:
  - keep samples refrigerated (2–8 °C);
  - ship samples at ≤ 2–8 °C;
  - formalin-fixed tissues can also be stored and shipped at room temperature; and
  - do not freeze whole blood samples.
- If the collection site has the required capacity, especially if samples cannot be shipped within 48 hours:
  - separate serum from whole blood;
  - freeze all samples, except fixed tissues; and
  - maintain the cold chain when shipping samples to the laboratory (ice packs, dry ice or liquid nitrogen).
- Preserving the sample integrity by maintaining the cold chain is critical for samples that will be used for direct virological methods.
- Samples for serological tests can be kept refrigerated (2–8 °C) for longer to facilitate transport.
- Freeze-thaw cycles should always be avoided.
2.4 Reception and handling of samples in the laboratory

Hygiene and biosafety conditions must be guaranteed in the sample reception areas. Once the samples are received in the laboratory, it is necessary to document their conditions at the time of receipt, and guarantee the traceability of the aliquots and the correct storage conditions. Protocols for detection and cleanup of biological sample spills are also essential.

2.4.1 Whole blood, serum/plasma, and other biological fluids

The following recommendations should be followed for whole blood, serum, and other biological fluids (urine, saliva, cerebrospinal fluid [CSF], amniotic fluid):

- Samples can be refrigerated (2–8 °C) if processed (or sent to a reference laboratory) within 48 hours.
- Total blood samples should be processed (i.e., serum or plasma preparation) upon arrival, and should not be frozen before processing.
- Samples received in the laboratory should be separated into several aliquots.
- Keep samples frozen (–10 to –20 °C) if processed after 48 hours.
- Keep samples frozen (–70 °C or less) if processed after 1 week.
- For long term storage, samples for serological methods might be kept frozen at –10 to –20 °C (or less) while samples for virological methods should be kept at –70 °C or less, if possible.
- Avoid freeze-thaw cycles.

2.4.2 Tissue samples

The following are recommendations for handling tissue samples:

- Immediately process (or freeze at –70 °C or less without any additive) fresh tissue samples for RNA extraction or virus isolation.
- Process or freeze tissue samples in RNA stabilizing solution, according to manufacturer’s instructions.
- Fixed tissue samples should be embedded in paraffin for histopathological and immunohistochemical studies. Formalin-fixed paraffin-embedded (FFPE) blocks are suitably stored at room temperature for extended periods.
- Fixed tissue samples and FFPE blocks can also be used for RNA extraction if fresh or frozen tissue samples are not available.

2.4.3 RNA

The following are recommendations for any handling of samples for RNA extraction or extracted RNA:

- Guarantee an RNase-free environment.
- Store the extracted RNA at –70 °C or less (RNA is stable at –70 °C for several months).
2.5 Shipment of samples to reference laboratories

To ship samples to reference laboratories, the following recommendations must be followed:

- Maintain the cold chain, preferably, with dry ice or refrigerant gels. Triple packaging should always be used (56).
- Samples should be shipped, preferably, within the first 48 hours of collection.
- Samples must be packaged, marked, appropriately labeled, and registered as category B (56, 57).
- Shipments must be accompanied by complete clinical and epidemiological records. For tissue samples, include the surgical or autopsy report.
- When shipping fixed tissue samples, FFPE blocks are preferable. FFPE blocks should be shipped at room temperature. If high temperatures are anticipated during transport, refrigerant gels can also be used (do not freeze).
- Fixed tissue samples should be packaged separately from fresh tissue and other samples, as formalin is volatile and might affect these samples.
- Shipments to reference regional laboratories and PAHO/WHO collaborating centers should be coordinated with the destination laboratory and the PAHO Regional Office.
3.1 Biosafety considerations

Laboratory personnel working with biological samples should be vaccinated against hepatitis B and yellow fever, and use appropriate personal protective equipment (PPE). Also, all necessary precautions to prevent percutaneous exposure should be taken. Fresh biological samples, regardless of sample type, should be considered potentially infectious (58, 59). Any procedure involving the handling of samples should be performed in certified class II biosafety cabinets (BSC), including the lysis step of RNA extraction (see section 4.1.1.1). Lysed samples are considered noninfectious although this might not be true for some viruses, such as the Ebola virus (60). Handling of extracted RNA does not require a BSC. For serological tests, the use of BSC and/or heat-inactivation of a sample aliquot (provided that the test protocol allows for it) should be considered. Fixed tissues also are generally considered non-infectious. Biosafety recommendations for virus isolation and neutralization assays depend on the strain of virus being cultured and national regulations (58). For neutralization assays, the use of vaccine or low pathogenicity strains is recommended.
3.2 Laboratory workflow

Nucleic acid amplification tests (NAAT), also known as molecular assays, are highly sensitive, and can detect minimum quantities (1–10 copies) of a nucleotide sequence of interest. This advantage is also a disadvantage, as DNA produced during the amplification process can contaminate laboratory areas, equipment, and supplies. Thus, laboratories must have dedicated areas for each step of the molecular assay set-up. Each area should have its own set of equipment, pipettes, and supplies. Reagents and materials should be nuclease-free, and surfaces, racks, cabinets, centrifuges, and micropipettes should be routinely cleaned and decontaminated with an RNase elimination reagent, UV irradiation and/or fresh 10% bleach solution followed by 70% ethanol. PPE should also be exclusive to each area. Three areas are usually warranted (61):

- Reagent preparation area (clean area) for reagent storage, and preparation of reagents and master mixes. No samples or positive controls should be handled in this area.
- Sample preparation area for the preparation of positive controls, nucleic acid extraction and addition of samples and positive controls to the master mixes.
- Amplification area for polymerase chain reaction (PCR) instruments.

The laboratory workflow should be unidirectional, from the reagent preparation area to the sample preparation area and finally to the amplification area; no backflow traffic of personnel (in the same day), samples, reagents, and supplies should be allowed (62–64). Some laboratories might further decrease the risk of contamination by having positive pressure in the reagent preparation area, and negative pressure in the remaining areas. Use of real-time PCR instruments avoids the handling of amplification products (amplicons), thus decreasing contamination risks. When end-point PCR is used, an additional area for electrophoresis, amplicon purification for DNA sequencing, and/or nested PCR is required. Extreme care should be taken to prevent contamination with nested PCR assays for which products from a first round of amplification serve as targets for a second round of amplification.
This chapter describes laboratory methods for the detection of DENV, CHIKV, ZIKV, and YFV infections, as well as test result interpretation. The general principle of each laboratory method is also described.

4.1 Virological diagnosis: direct methods

Virological methods allow the direct identification of the virus or one of its components. The main virological methods are the detection of genome sequences, the detection of antigens, and virus isolation. These methods are described below.

4.1.1 Molecular methods: amplification and detection of the viral genome

Several methods have been developed for the amplification and subsequent detection of specific nucleic acid sequences. These nucleic acid amplification tests are, for the most part, based on PCR. Other techniques, such as reverse transcription loop-mediated isothermal amplification (RT-LAMP) and transcription-mediated amplification (TMA), also allow genome amplification, and can be used for molecular diagnosis, e.g., for the detection of ZIKV (65, 66).

4.1.1.1 RNA extraction

Molecular methods usually require that nucleic acids are extracted from the sample and purified prior to amplification. Bodily fluids such as serum, plasma, urine, saliva, and CSF are directly lysed and extracted,
while tissue samples (fresh, formalin-fixed tissue samples, or preserved in RNA stabilizing solutions) require prior disruption and homogenization. Sections from FFPE blocks should be deparaffinized with xylol (or other solvents) before disruption and homogenization; the latter can be achieved by enzymatic or mechanical procedures. Depending on sample type, a variety of commercial kits and in-house protocols are available for deparaffinization, disruption, homogenization, lysis and RNA purification. For RNA extraction, supplies and reagents should be RNase-free, all surfaces and equipment involved in the process should be thoroughly cleaned, and powder-free gloves should be used.

4.1.1.2 Reverse transcription
As viruses discussed in this document have an RNA genome, synthesis of a complementary DNA (cDNA) from the viral RNA is required, prior to amplification by PCR. This is achieved by reverse transcription (RT), and the combination of both techniques is called reverse transcription polymerase chain reaction (RT-PCR).

Two approaches can be used for RT-PCR. The first approach requires obtaining cDNA through an independent RT step, for which random hexamers, sequence-specific primers, or oligo-dT primers (for viruses with poly(A) tails) can be used. If testing for different viral agents, random hexamers are preferred, as they allow reverse transcription of all RNAs present in the sample. An aliquot of cDNA is then used as a template for virus-specific PCR; the entire procedure is known as two-step RT-PCR. Another approach is a one-step RT-PCR, for which a mix of a reverse transcriptase and a hot start DNA polymerase is used in a single tube for sequence-specific cDNA synthesis and subsequent PCR amplification. This option is often recommended for diagnostic purposes, as it does not require cDNA manipulation, therefore decreasing the risk of contamination.

4.1.1.3 Reverse transcription polymerase chain reaction
RT-PCR can be end-point (also known as conventional RT-PCR, in which the final amplification products are separated and identified according to their size by electrophoresis on agarose or polyacrylamide gels) or real-time (monitoring of the amplification throughout the reaction using fluorescence). Some of these assays allow simultaneous detection of several arboviruses (multiplex assays).

Reference RT-PCR assays for detecting DENV, CHIKV, ZIKV, and YFV are described in Table 2. The list does not restrict the assays that can be used for molecular detection of these viruses. Other tests can be used, as long as they have been rigorously evaluated against reference tests. It should be noted that harmonization of tests used in the Region of the Americas facilitates the comparison of national data, as well as the technical support that PAHO and collaborating centers can provide to public health laboratories.
### TABLE 2  
**Reference molecular assays for the detection of ribonucleic acid from dengue, chikungunya, Zika, and yellow fever viruses**

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>TYPE OF ASSAY</th>
<th>TARGET SEQUENCE</th>
<th>COMMENTS</th>
</tr>
</thead>
</table>
| DENV, CHIKV and ZIKV<sup>1</sup> | Real-time multiplex RT-PCR | DENV: 5' UTR  
CHIKV: nsP1  
ZIKV: Envelope | CDC Trioplex Real-time RT-PCR for the simultaneous detection of the three viral RNAs |
| DENV<sup>2</sup> | Real-time multiplex RT-PCR | DENV-1: NS5  
DENV-2: Envelope  
DENV-3: prM  
DENV-4: prM | CDC DENV-1-4 Real-Time RT-PCR for the simultaneous detection and typing of the four DENV types |
| CHIKV<sup>3</sup> | Real-time singleplex RT-PCR | nsP1  
nsP2 | Primers/probe: CHIK856F / CHIK962C / CHIK908FAM  
Primers/probe: CHIKV3855F / CHIKV3957C / CHIKV3886 FAM |
| ZIKV | Real-time singleplex RT-PCR | Envelope<sup>4</sup>  
NS2b<sup>a</sup> | Use only the primers/probe: ZIKV 1087 / 1163c / 1108FAM (previously named ZIKV 1086 / 1162c / 1107FAM)  
ZIKV 4481 / 4552c / 4507cFAM primers/probe can also be used. |
| YFV<sup>b</sup> | Real-time singleplex RT-PCR | 5' UTR<sup>5</sup>  
5' UTR<sup>a</sup> | Primers/probe: YFallF / YFallR / YFallP  
Primers/probes: YF14-34 / YF115C / YF34-57FAM |

Notes:  
<sup>a</sup> Arboviral Diseases Branch, United States Centers for Disease Control and Prevention and PAHO Regional Office in Washington, DC. The sequences and protocols are available at: https://www.paho.org/es/temas/dengue/red-laboratorios-diagnostico-arbovirus-relda.  
<sup>b</sup> These protocols detect all virus strains, including the YFV17D vaccine strain. For the specific detection of the YFV17D strain, the protocol of Hughes et al. can be considered: Hughes HR, Russell BJ, Mossel EC, Kayiwa J, Lutwama J, Lambert AJ. Development of a real-time reverse transcription-PCR Assay for global differentiation of yellow fever virus vaccine-related adverse events from natural infections. J Clin Microbiol. 2018;56(6).  
<sup>c</sup> CDC: Centers for Disease Control and Prevention of the United States of America; CHIKV: chikungunya virus; DENV: dengue virus; NS: nonstructural protein; prM: premembrane protein; RT-PCR: reverse transcription polymerase chain reaction; UTR: untranslated region; YFV: yellow fever virus; ZIKV: Zika virus.  

References:  
4.1.1.4 Dynamics of the molecular detection of arbovirus in different biological samples
Thanks to the amplification process, molecular assays are highly sensitive, and, as long as the region of the amplified viral genome differentiates the target virus from other genetically related viruses, these assays are also highly specific. However, actual sensitivity of molecular assays depends on the levels of the specific viral RNA to be detected in a specific biological sample, and the temporal dynamics of these levels with respect to the onset of the disease symptoms. Several examples can be cited, all of which impact the sensitivity of molecular detection: decreasing DENV, CHIKV, ZIKV, and YFV viremia with time since symptom onset; lower ZIKV viremia when compared to DENV or CHIKV (67); higher ZIKV RNA levels in urine versus serum (4). The period in which DENV, CHIKV, ZIKV, and YFV might be detected in biological samples is described in Table 3. Importantly, the date of onset of symptoms is usually reported by the patient and might be subject to bias, particularly in Zika cases which are often paucisymptomatic. Finally, sample quality is a key factor affecting molecular assay sensitivity.

TABLE 3  Periods of molecular detection of arboviruses in biological samples

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>TYPE OF SAMPLE</th>
<th>DAYS AFTER SYMPTOM ONSET</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV1</td>
<td>Serum1</td>
<td>1–7</td>
</tr>
<tr>
<td>CHIKV2</td>
<td>Serum2</td>
<td>1–8</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Serum3</td>
<td>1–4 to 5b</td>
</tr>
<tr>
<td></td>
<td>Urine3</td>
<td>1–15b</td>
</tr>
<tr>
<td></td>
<td>Whole blood4–6</td>
<td>1–28</td>
</tr>
<tr>
<td></td>
<td>CSF5–3</td>
<td>Any dayd</td>
</tr>
<tr>
<td></td>
<td>Saliva3</td>
<td>1–4 to 5</td>
</tr>
<tr>
<td>YFV</td>
<td>Serum7</td>
<td>1–10</td>
</tr>
</tbody>
</table>

Notes:  
1 In general, a decrease in DENV, CHIKV, ZIKV, and YFV viremia is observed with time from symptom onset, which may affect the sensitivity of molecular detection.  
3 Only if collected for clinical reasons. Do not collect the sample for the sole purpose of identifying the etiological agent.  
4 Although the suspicion of neurological syndrome could occur outside the detection period, attempting molecular detection in available samples is recommended (see section 4.3).  
6 Lustig Y, Mendelson E, Paran N, Melamed S, Schwart E. Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016. Euro Surveill. 2016;21(26).  

Sources:  
5 Lustig Y, Mendelson E, Paran N, Melamed S, Schwart E. Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016. Euro Surveill. 2016;21(26).  
4.1.1.5 Molecular methods for the viral diagnosis of fatal cases

Infection with YFV, as well as with DENV and CHIKV (and ZIKV in some cases), can be fatal. Therefore, it is important to confirm or rule out these cases postmortem through laboratory testing. Besides the samples described in Table 3, molecular methods can be used to detect viral RNA in tissues. Table 4 presents a list of tissues that can be used for molecular diagnosis in fatal cases suspected of yellow fever, dengue, chikungunya, or Zika.

4.1.1.6 Generic molecular detection methods

Some RT-PCR assays are designed to detect the viral genus taking advantage of conserved genetic sequences among viruses of the same genus. For instance, generic assays have been designed for the detection of viruses of the genera *Flavivirus*, *Alphavirus*, *Peribunyavirus*, and *Phlebovirus* (also called pan-flavivirus, pan-alphavirus, pan-peribunyavirus, and pan-phlebovirus assays) (68–75). Currently, these assays have some limitations among which are that they are end-point RT-PCRs (and sometimes nested RT-PCRs); their sensitivity varies, depending on the virus to be detected, and is generally lower than virus-specific RT-PCRs; and they have not been sufficiently evaluated by PAHO/WHO Collaborating Centers. After a positive test result using a generic RT-PCR, the identification of the infecting virus requires virus-specific RT-PCRs, or amplification product sequencing (see Chapter 7).

These assays may be useful for a more extensive screening of viruses potentially present in the sample. In a suspected case, a combination of generic RT-PCRs might be used to identify the genus of the etiologic

| TABLE 4 | Samples for the molecular detection of dengue, chikungunya, Zika, and yellow fever viruses in fatal cases |
|-----------------|-------------------------------------------------|-------------------------------------------------|
| VIRUS           | RECOMMENDED SAMPLES                             | OTHER SAMPLES                                    |
| DENV<sup>1,2</sup> | Liver                                           | Spleen, kidney, lung, lymph nodes, thymus, bone marrow, CSF,<sup>a</sup> brain,<sup>a</sup> |
| CHIKV<sup>3</sup>    | b                                                | Serum, CSF,<sup>a</sup> brain,<sup>a</sup> any available tissue sample<sup>b</sup> |
| ZIKV<sup>4</sup>     | Brain, liver, kidney, and placenta              | –                                               |
| YFV<sup>5</sup>      | Liver, kidney                                   | Spleen, brain, lung, heart, lymph nodes         |

Notes:  
<sup>a</sup> Useful in cases with suspected encephalopathy or encephalitis due to DENV or CHIKV.  
<sup>b</sup> No particular sample recommended. Detection should be attempted on any available sample.

Sources:  
agent, followed by virus-specific RT-PCRs or nucleotide sequencing. Generic assays can also be used to screen surveillance samples that have yielded negative results for clinically suspected virus(es). In such cases, viruses that have not been previously characterized in the Region might be identified, as was the case in the identification of Punta Toro virus species complex (genus *Phlebovirus*, family *Phenuiviridae*) in Panama (76).

### 4.1.2 Detection of viral antigens

Detection of viral antigens, namely proteins or glycoproteins, depends mainly on their presence and abundance in biological samples. The best arboviral antigen for laboratory detection in serum is the DENV NS1 protein. Detection of viral immunohistochemical antigens in tissue sections is also useful in fatal cases suspected of arboviral infection, in particular yellow fever cases.

#### 4.1.2.1 Detection of DENV NS1 protein by enzyme-linked immunosorbent assay

NS1 is a nonstructural DENV protein secreted by infected cells. It can be detected in serum, both in primary and secondary infections; the detection period is longer in the former infections (i.e., the first time a person is infected with DENV) than in the latter (i.e., subsequent DENV infections) (Figure 1). Consistently, the sensitivity of detection is lower in secondary infections. Generally, concentration of the NS1 protein in blood decreases during infection and its detection sensitivity decreases accordingly. Although NS1 temporal kinetics differs between primary and secondary DENV infections, whether the patient is exhibiting an acute primary or a secondary infection is generally not known when testing samples. NS1 detection sensitivity also varies between DENV serotypes (77–82).

The DENV NS1 protein can be detected in the laboratory by enzyme-linked immunosorbent assay (ELISA). Rapid immunochromatographic tests for detection of NS1 are available, although they are less sensitive than ELISA and their use in reference laboratories is generally not recommended. Several NS1 ELISA and rapid tests have been evaluated in the Region and their sensitivity and specificity established (78, 83). However, these evaluations were performed prior to the introduction of ZIKV in the Region, and their specificity must be reassessed in the current epidemiological context of co-circulation of multiple flaviviruses.

To date, antigens for CHIKV, ZIKV, or YFV detection in serum or other sample types have not been validated.

#### 4.1.2.2 Detection of viral antigens by immunohistochemistry

In fatal cases of arbovirus infections, antigen detection in tissue sections by immunohistochemistry (IHC) or immunofluorescence can be used for diagnosis confirmation. Antigens are detected using specific antibodies, which can be conjugated to a fluorophore or an enzyme for detection (direct method) or be detected with a conjugated secondary antibody (indirect method). These methods are generally combined with histopathological analysis to identify histological alterations typical of some arboviruses, such as Councilman bodies in the liver of yellow fever cases. Currently, IHC is mostly used for YFV antigen detection, in which case, liver and kidney are the tissues of choice (84). Spleen, brain, lung,
heart and lymph node samples may also be useful (Table 5). The use of well-characterized monoclonal antibodies (e.g., 2D12) (85) is recommended. IHC has also been used to diagnose fatal cases of dengue (86, 87) and chikungunya (5) (Table 5).

4.1.2.3 Potential cross-reactivity in viral antigen detection tests

Detection of viral antigens is based on the use of antibodies, and, given antigen similarity among viruses of the same genus, the potential for cross-reactivity of these assays needs to be taken into account. Cross-reactivity could impact assay specificity. In particular, the performance of DENV NS1 ELISAs and rapid tests should be reevaluated in the context of multiple flaviviruses co-circulation (e.g., DENV, ZIKV, and YFV). To date, two studies have reported a high specificity of DENV NS1 ELISA tests in laboratory-confirmed Zika cases (88, 89).

Monoclonal antibodies for IHC and immunofluorescence should also be carefully selected and evaluated to ensure specificity.

4.1.3 Virus isolation

Virus isolation is primarily performed in cell culture, or by inoculation of suckling mice and other rodents. Mammalian cell lines (notably, Vero cell cultures) are often used, as well as mosquito cells (e.g., C6/36 cell cultures) (86, 90, 91). A wide variety of samples can be used for arbovirus isolation including serum, total blood, CSF, human or animal tissues, and mosquitoes. In fatal cases, tissue samples must be collected within 24 hours of death.

In most cases of arboviral infections, virus isolation is not routinely used for diagnosis, nor is it a requirement for confirmation. Technical complexity (including the need for infrastructure for the preparation and maintenance of cell cultures and/or animal facilities), costs, biosafety requirements for some viruses, as well as the need to identify isolated viruses by RT-PCR or immunofluorescence, limit the use and timeliness of virus isolation for diagnosis. However, it is important to isolate strains that can then be genetically and phenotypically characterized, and serve as reference reagents and controls for serological and molecular assays. In addition, virus isolation may allow the identification of new viruses, making it an important tool for surveillance of emerging and reemerging viruses.

### TABLE 5

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>RECOMMENDED TISSUE</th>
<th>OTHER TISSUE SAMPLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>YFV</td>
<td>Liver, kidney</td>
<td>Spleen, brain, lung, heart, and lymph nodes</td>
</tr>
<tr>
<td>DENV</td>
<td>Liver</td>
<td>Spleen, kidney, lung, lymph nodes, thymus, bone marrow, and brain&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CHIKV</td>
<td>Brain&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Any other available tissue sample&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Notes: <sup>a</sup> Useful in cases with suspected encephalopathy or encephalitis due to DENV or CHIKV.  
<sup>b</sup> No particular sample recommended. Detection should be attempted on any available sample.  
CHIKV: chikungunya virus; DENV: dengue; YFV: yellow fever virus.
4.2 Serological diagnosis: indirect methods

Serological methods are based on the detection of the immune response to viral infection, particularly the antibody response that is mainly detected in serum (or CSF in neurological diseases). The sensitivity of serological methods is mainly dictated by the temporal dynamics of antibody production. These dynamics have been well characterized for DENV infection (55, 92). In primary infections, anti-DENV IgM antibody levels rise gradually during the first week after symptom onset, while IgG antibodies are first detectable 5–7 days after the onset of symptoms (see Figure 1). In contrast, during secondary DENV infections, IgG antibodies are produced earlier and reach higher levels, while IgM antibody levels are usually lower than in primary infections (92, 93). IgM antibodies wane in the weeks or months following infection, although longer persistence has also been described depending on the infecting virus. IgG antibodies are long-lasting and can be detected for life.

4.2.1 Detection of immunoglobulin M antibodies

Immunoglobulin M (IgM) antibodies against different arboviruses can be detected by ELISA (mainly using IgM capture ELISA [MAC-ELISA]) or other immunoassays, such as indirect immunofluorescence assays (IFA). Several laboratories have developed their own or “in-house” MAC-ELISA procedures using inactivated virus or recombinant viral proteins as antigens. MAC-ELISAs developed at PAHO/WHO Collaborating Centers include MAC-ELISAs for ZIKV, DENV, CHIKV, and YFV of the US Centers for Disease Control and Prevention (CDC) (5, 94–96), and the DENV MAC-ELISA (52, 86) of the Instituto de Medicina Tropical Pedro Kourí (IPK, Cuba) (Table 6). Moreover, kit versions of the CDC YFV MAC-ELISA

![Figure 1](https://www.cdc.gov/dengue/training/cmerccm/page53677.html)
and the IPK DENV MAC-ELISA for easier implementation have been optimized (86, 94) (Table 6). In addition, several commercial kits for detecting IgM antibodies are available. The United States Food and Drug Administration (FDA) has authorized several ELISA tests to detect DENV and ZIKV IgM (97–99). The performance of several commercial kits has also been evaluated with well-characterized sets of samples by laboratories in the Region, in particular for DENV (83, 100, 101), CHIKV (102), and ZIKV (103, 104) (Table 6). However, it is important to note that the evaluations of DENV kits were conducted before the introduction of ZIKV in the Americas, and that cross-reactivity with ZIKV was not explored (83, 100, 101). Thus, additional evaluations are needed to determine the specificity of these kits in ZIKV infections. As for molecular methods, this list of reference assays does not restrict the development and use of other assays. Nonetheless, those other assays should be evaluated before they are used for routine surveillance.

IgM is detected in serum or in CSF in cases of neurological disease with available samples. In cases of neurological disease, it is recommended to process serum and CSF samples in parallel. The CSF sample is assayed pure or at low dilution (maximum dilution of 1/5). The presence of IgM in the CSF confirms a recent infection of the central nervous system, always considering the potential persistence of IgM antibodies and the potential cross-reactivity between viruses of the same genus (see section 4.2.4).

### 4.2.2 Detection of immunoglobulin G antibodies

Immunoglobulin G (IgG) antibodies are usually detected by ELISA, although other techniques, such as immunofluorescence, complement fixation, and hemagglutination inhibition assays, as well as microsphere immunoassays, may be used. As IgG antibodies to the virus are long-lived, the diagnostic value of IgG measurements in a single sample is limited. Confirmation of the infection requires the detection of IgG seroconversion or a fourfold or greater increase in IgG titers between the acute and convalescent samples (94). The latter requires a quantitative IgG test, while most commercial IgG assays are qualitative. Moreover, cross-reactivity of IgG antibodies to viruses of the same genus further limits the use of IgG detection (see section 4.2.4, Limitations of serological methods). However, recent studies suggest that antibodies to the NS1 protein have a better specificity than antibodies to the envelope, which may help the diagnosis (103, 105, 106).

### 4.2.3 Detection of neutralizing antibodies

The detection of neutralizing antibodies using the plaque reduction neutralization test (PRNT) is considered the gold standard for serologic detection of arboviral infections (107). PRNT, and other neutralization assays, are based on the ability of serum antibodies from infected persons to inhibit (namely, neutralize) virus infection in cell cultures. Serial dilutions of serum are used to calculate the titer required to reduce the number of viral plaques by a certain percentage (e.g., by 90% for PRNT<sub>90</sub>). PRNT requires cell culture facilities and is resource- and time-intensive. A wide range of cell lines, viral strains, and other assay conditions and analysis methods have been reported; thus, the standardization of the conditions of this test is essential (107–109).
TABLE 6  Reference IgM assays for the detection of dengue, chikungunya, Zika, and yellow fever virus infections

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>TYPE OF SAMPLE</th>
<th>PAHO/WHO COLLABORATING CENTER ASSAYS</th>
<th>FDA APPROVED ASSAYS</th>
<th>EVALUATIONS PERFORMED BY PAHO/WHO COLLABORATING CENTERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV</td>
<td>Serum, CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CDC dengue MAC-ELISA&lt;sup&gt;1&lt;/sup&gt;</td>
<td>4</td>
<td>5–7,b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IPK dengue MAC-ELISA&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHIKV</td>
<td>Serum, CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CDC CHIKV MAC-ELISA&lt;sup&gt;8&lt;/sup&gt;</td>
<td>–</td>
<td>9</td>
</tr>
<tr>
<td>ZIKV</td>
<td>Serum, CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CDC Zika MAC-ELISA&lt;sup&gt;10,11&lt;/sup&gt;</td>
<td>11,12</td>
<td>13,14</td>
</tr>
<tr>
<td>YFV</td>
<td>Serum, CSF&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CDC YF MAC-ELISA&lt;sup&gt;1,15&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDC YF MAC-HD&lt;sup&gt;15&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notes:  
<sup>a</sup> Test only if collected for clinical reasons. Do not collect CSF for the sole purpose of determining the infectious agent.  
<sup>b</sup> These evaluations were carried out before the introduction of ZIKV in the Region; cross-reactivity with this virus was not assessed.  
CDC: Centers for Disease Control and Prevention of the United States of America; CSF: cerebrospinal fluid; CHIKV: chikungunya virus; DENV: dengue virus; FDA: United States Food and Drug Administration; IPK: Instituto de Medicina Tropical Pedro Kouri (Cuba); MAC-ELISA: IgM antibody capture enzyme-linked immunosorbent assay; PAHO/WHO: Pan American Health Organization/World Health Organization; YFV: yellow fever virus; ZIKV: Zika virus.

Sources:  
A seroconversion or a fourfold or greater increase in titers between the acute and the convalescent phase samples is usually required for confirmation of the infecting agent. Nevertheless, within-genus neutralizing antibody cross-reactivity has been reported, particularly in persons experiencing a secondary flavivirus infection, in whom a simultaneous rise in neutralizing antibody titers to multiple flaviviruses can be observed (95, 110). For this reason, it is recommended to perform the simultaneous detection of neutralizing antibodies against a flavivirus panel containing the relevant viruses according to the epidemiological context. However, in secondary infections, the increase in titers against the virus that caused the primary infection may be greater than the increase in titers against the virus that causes the secondary infection, a phenomenon known as the original antigenic sin (111). This phenomenon further hampers the interpretation of results.

### 4.2.4 Limitations of serological methods

The use of serological techniques has several limitations. First, a positive IgM test result in a single sample is only presumptive of an acute infection, since the antibodies detected may come from a recent infection and not necessarily from the acute infection. In the case of diseases for which there is a vaccine, detected antibodies may also come from a recent vaccination. The persistence of IgM antibodies has not been fully characterized for DENV, CHIKV, ZIKV, and YFV infections, and some data suggest persistence might be longer than initially thought (112–114). Detection of IgG antibodies, which persist longer than IgM antibodies, in a single sample only allows provisional interpretation. Laboratory confirmation of an acute infection requires paired serum samples: acute (usually collected during the first week after symptom onset) and convalescent (ideally collected at least 2 weeks after the acute sample). Seroconversion of IgM (negative acute and positive convalescent sample) or an increase in IgG or neutralizing antibody titers between the two samples (a fourfold or greater increase in antibody titers) is considered confirmatory of the acute infection. However, the confirmation of the etiologic agent is limited by the cross-reactivity of serological assays following infections by and/or vaccination against viruses of the same genus.

This cross-reactivity is higher in secondary than in primary infections. Thus, in areas with current or previous co-circulation of several flaviviruses (the current epidemiological context in most of the Americas), the probability of cross-reactivity is high. Cross-reactivity between different alphaviruses has also been reported, although this has not been as extensively characterized as in flaviviruses. Therefore, samples with a positive IgM result for a particular flavivirus or alphavirus must be analyzed by differential IgM assays. Differential tests to be conducted should be based on the epidemiological context of the case’s area of residency and/or exposure. It is important to emphasize that cross-reactivity in serological tests depends on the test’s design. Currently, more specific assays are being developed. In general, neutralization assays offer greater specificity than IgM or IgG antibody detection. However, cross-reactivity among flaviviruses has also been documented in neutralization assays (95, 115), and the performance of differential tests with a flavivirus panel is recommended. As mentioned above, particularly for secondary infections, it might be impossible to determine the infecting flavivirus even when parallel testing for several viruses.
Finally, the limited availability of commercial kits with independent evaluations, and the limited supply of reagents for reference in-house assays restricts the use of serological methods.

### 4.2.5 Use and interpretation of serological methods

Despite their limitations, serological tests are part of the toolbox for diagnosing arboviral infections for several reasons: 1) the use of virological methods depends on the availability of high-quality samples collected in the right time window; 2) the patient may present after the viremic phase has ended; 3) virological methods are not always available, as they require specific laboratory areas and equipment. In addition, given the costs associated with extraction and amplification reagents, serological methods (especially in-house ELISA tests), which are significantly cheaper and simpler to perform than virological methods, might be easier to implement in a subnational network of laboratories; 4) the combined use of virological and serological methods may increase diagnostic sensitivity and specificity (84, 116); and 5) serological methods are essential when the virus and its components are rarely found in biological samples, such as in newborns with congenital Zika syndrome or, to a lesser extent, in patients with neurological syndromes associated with ZIKV or other arboviruses.

When only serological assay results are available from the laboratory, particular caution should be exercised in their interpretation. For instance, if serological methods on single samples are used for routine surveillance of a particular virus, the trends observed might be caused by a different virus from the same genus, and results should be interpreted as such, until additional evidence is gathered. Health care providers, as well as patients through pre- and post-test counseling, should also be aware of these limitations to prevent the misinterpretation of results. Importantly, the integration of laboratory, clinical, and epidemiological information, and their analysis by multidisciplinary teams at local and national levels, should be a priority for the interpretation of results, and, when necessary, for alert and response.

### 4.3 Recommended algorithms for laboratory diagnosis of dengue, chikungunya, Zika, and yellow fever virus infections

Recommended diagnostic algorithms include molecular and serological methods. The algorithms are used for the diagnosis of the patient and for epidemiological and laboratory-based surveillance. They allow monitoring of a specific epidemiological situation and the consequent implementation of prevention and control actions. The algorithms are meant as a guide for national authorities and reference laboratories, and should be adapted to each place and epidemiological situation dynamically.

When implementing an algorithm, the following must be considered: 1) the epidemiological situation of the area or country, which may vary due to environmental, climatic, and demographic factors, vaccination policies and health policies in general, circulating viruses, immune history of the population, among others; 2) the laboratory capacity to implement the recommended algorithms at both national and local levels; 3) the availability of necessary tests and reagents that may be limited; and 4) the specific epidemiological situation (an outbreak of a known virus, the emergence of a new virus into the
area or country, the co-circulation of several viruses) which may affect the application and interpretation of laboratory algorithms. Therefore, the recommended algorithms described below should be adapted in each country to the needs of epidemiological surveillance and to the laboratory capacity. In addition, it is important to integrate laboratory results with clinical, epidemiological, and environmental information, to allow for proper characterization of the epidemiological situation.

Given the limitations of the aforementioned serological methods, the use of virological methods, particularly molecular ones, should be prioritized whenever possible, always taking into consideration cost and effectiveness. In addition, the collection of paired acute and convalescent samples in a subset of cases should be implemented. This approach may facilitate the etiologic agent identification by serology and is essential to evaluate the diagnostic performance of serological tests. In the context of a new outbreak, collection of paired samples is also particularly relevant. Finally, in the current epidemiological scenario, with the co-circulation of many alphaviruses and flaviviruses in the Region, the study of immune responses in paired samples with serological assays, combined with molecular detection of the etiologic virus, might often be required for a full characterization of outbreaks.

4.3.1 Suspected cases of dengue, chikungunya, and Zika
The algorithms for virological and serological testing of suspected dengue, chikungunya, and Zika cases (Figures 2 and 3) have been previously published (4).

4.3.1.1 Virological testing
Depending on clinical presentation, epidemiological context, and availability of multiplexed RT-PCR assays, the detection of DENV, CHIKV, and ZIKV might be attempted sequentially or in parallel. As indicated in Figure 2, during the acute stage of infection serum is the preferred sample for routine RT-PCR testing of DENV, CHIKV and ZIKV. However, urine and whole blood can also be used for acute ZIKV testing. While ZIKV typically shows a low and short viremia, DENV and CHIKV might be detected in serum for up to 7 and 8 days, respectively. Periods of likely presence of the viral RNAs in different sample types are summarized in Table 3. During the acute phase, DENV NS1 antigen testing can also be used (in this case, only a serum sample is suitable). Finally, negative RT-PCR results might be due to a resolved viremia and serological testing might be attempted in these cases.

4.3.1.2 Serological testing
A serum sample is required for serological testing. As discussed in sections 4.2.4 on the limitations of serological methods and 4.2.5 on the use and interpretation of serological methods, a positive IgM test in a single sample is not confirmatory of an acute infection. Depending on the epidemiological context, other flaviviruses than DENV and ZIKV might need to be included in the differential diagnosis. In the absence of other laboratory results, positive testing for more than one flavivirus IgM is interpreted as a recent flavivirus infection. Testing for neutralizing antibodies (performed ideally in paired acute and convalescent samples) may resolve cross-reactive results. Of note, IgM may also not be present or below detection limits for some secondary flavivirus infections.
**FIGURE 2** Algorithm for virological testing of suspected cases of dengue, chikungunya, and Zika

Serum sample obtained within 5 days of symptom onset

- **ZIKV**
  - ZIKV RT-PCR
  - Positive
  - Negative

- **DENV**
  - DENV RT-PCR
  - Positive
  - Negative

- **CHIKV**
  - CHIKV RT-PCR
  - Positive
  - Negative

Note: CHIKV: chikungunya virus; DENV: dengue virus; RT-PCR: reverse transcription polymerase chain reaction; ZIKV: Zika virus.


**FIGURE 3** Algorithm for serological testing of suspected cases of dengue and Zika

Serum sample obtained 6 days after symptom onset or later

- **IgM (ELISA)**
  - DENV positive
    - ZIKV negative
    - Probable DENV
  - DENV positive
    - ZIKV positive
    - Probable infection
  - DENV negative
    - ZIKV positive
    - Probable ZIKV

Note: DENV: dengue virus; ELISA: enzyme-linked immunosorbent assay; ZIKV: Zika virus.

CHIKV infection is not included in the algorithm as alphavirus serology shows substantially less within-family cross-reactivity than flavivirus serology. However, depending on the epidemiological context, differential diagnosis with other alphaviruses might be needed.

4.3.1.3 Special considerations for suspected cases of Zika virus-associated Guillain-Barré syndrome and other neurological syndromes

In these cases, blood and urine samples should be collected and processed by molecular methods, regardless of the time since the onset of symptoms. If collected for clinical reasons, CSF samples should also be tested by molecular methods. The highest sensitivity for molecular methods has been reported in urine, with approximately 60% of positive tests, while in serum/plasma and CSF sensitivity is low (approximately 5%) (117–123). Although a positive molecular test is confirmatory of ZIKV infection, a negative result does not rule it out, and serological testing should then be performed. To this end, both serum and CSF might be used and interpreted according to the general algorithms (Figures 2 and 3). Any ZIKV IgM positive result is highly suggestive of a ZIKV-associated case, as DENV-associated Guillain-Barré syndrome (GBS) is less common. However, because of the potential persistence of IgM antibodies, other causes of GBS should be considered and ruled out before determining that GBS is associated with a recent ZIKV infection (4).

4.3.1.4 Special considerations for suspected cases of Zika virus-associated microcephaly and other congenital syndromes

ZIKV infection in pregnant women can be detected based on the recommended algorithms in Figures 2 and 3, above. The period of detection in both serum and urine has been shown to be longer in pregnant women (124). ZIKV is also detectable in amniotic fluid. At birth, placental tissue should be collected, as well as serum and urine from the newborn (124). If umbilical cord blood is collected, extreme caution should be used to prevent contamination of the sample with maternal blood. Newborn blood and urine should be collected within 2 days of birth. Molecular ZIKV detection in placenta, blood, and urine (and CSF, if collected for clinical reasons) should be attempted. However, most tests are negative at birth. Thus, serological testing is essential in molecular test-negative cases. Because ZIKV intraterine infection would likely constitute the newborn’s primary flavivirus infection, detection of ZIKV IgM antibodies at birth is considered to be highly indicative of a ZIKV infection (4). When ZIKV congenital infection is suspected, laboratory tests to rule out other infections (e.g., cytomegalovirus, herpes simplex virus, rubella, HIV, toxoplasmosis, and syphilis) are also recommended (4). It is important to highlight the combined analysis serological results from the mother and the newborn to facilitate their interpretation. For instance, a negative IgG test or PRNT for ZIKV in the mother at the time of delivery rules out infection by this agent.

4.3.2 Suspected cases of yellow fever

The algorithms for laboratory confirmation of yellow fever cases (Figure 4) including fatal cases (Figure 5) are presented and further discussed in PAHO’s recommendations for laboratory diagnosis of yellow fever virus infection (84).
Algorithm for laboratory confirmation of yellow fever (YF) cases

Suspected YF case

≤10 days from symptom onset vs. Sample collection

YF RT-PCR2,3

Positive

Confirmed YF case

Negative

YF IgM ELISA3

Positive

IgM differential diagnosis4

Negative

Recent flavivirus infection5

Sample collected ≤7 days from symptom onset

Probable YF case6

Sample collected ≥8 days from symptom onset

Inconclusive7

Exclude YF8

Notes:

1 No YF vaccination within 30 days or unknown YF vaccination history.
2 Laboratories that only have the capacity to perform RT-PCR or IgM ELISA should test samples with the available technique. Results should be interpreted according to the algorithm.
3 RT-PCR sensitivity is higher in the first 10 days from symptom onset. However, detection up to 14 days has been reported, in particular in severe (and fatal) cases.
4 Must include dengue virus as well as other flaviviruses depending on the epidemiological situation of the area/country.
5 Consider performing PRNT in a reference laboratory. This result does not rule out yellow fever. Thus, in areas where no YF circulation has been described recently, this should prompt an investigation.
6 A positive IgM test in a single sample is not confirmatory. Additional clinical and epidemiological criteria must be used for the final interpretation of the case, in particular in areas where no YF circulation has been described recently.
7 A second sample should be requested and tested according to the algorithm.
8 Cases should be investigated and clinical differential diagnosis performed.

Algorithm for laboratory confirmation of fatal yellow fever (YF) cases

1. Fatal case suspected of YF

   - Fixed tissue samples
   - YF immunohistochemistry
     - Negative: Exclude YF
     - Positive: Confirmed YF case

   - Serum and fresh tissue samples
   - YF RT-PCR
     - Positive: Confirmed YF case
     - Negative: Serum sample

   - YF IgM ELISA

Notes:
1. No YF vaccination within 30 days or unknown YF vaccination history.
2. Fresh and fixed tissue samples should be collected (in particular, liver and kidney).
3. Immunohistochemistry should be performed on liver and kidney sections and, if available, on other tissue samples.
4. Cases should be investigated and clinical differential diagnosis performed.
5. RT-PCR should be performed on RNA extracted from serum and fresh tissue samples.
6. Follow the interpretation described in the algorithm for laboratory confirmation of YF cases (Figure 2).

Laboratory diagnosis of Mayaro and Oropouche virus infections

5.1 Mayaro virus infection

Mayaro fever is caused by Mayaro virus (MAYV, genus Alphavirus, family Togaviridae). MAYV is part of the Semliki Forest virus complex, a serological alphavirus group that may present immunological cross-reactivity among its members, and that notably includes CHIKV. This, together with the nonspecific nature of MAYV symptoms and the endemicity of different arboviruses in the Region of the Americas, may lead to misdiagnosis.

Laboratory assays to detect MAYV include viral RNA detection in serum by RT-PCR (125–127), and viral isolation (128–130). The latter can be performed by inoculating patient serum, total blood, or tissues into cultures of C6/36 or Vero cells, or by the intracerebral inoculation of suckling mice. However, the infection’s short viremia period (usually up to 6 days after symptom onset) limits the use of these techniques. On the other hand, IgM against MAYV is produced 3 to 5 days after symptom onset, and can persist for up to 3 months, while IgG antibodies persist for years (128–130). Different in-house serological assays have been used for detection of antibodies against MAYV, such as ELISA, immunofluorescence, hemagglutination inhibition, complement fixation, or neutralization tests (128–130). These assays, including IgM ELISA, are subject to cross-reactivity with other alphaviruses, in particular those within the Semliki Forest virus complex, but to a lesser extent than for flaviviruses (131) (see also section 4.2.4, Limitations of serological methods). MAYV induces lysis cell plaques in Vero or BHK-21 cells which allows for the use of PRNT. PRNT is considered the gold standard of
serological testing for MAYV infection, although cross-reactivity may also occur. Confirmation by serology usually requires paired acute- and convalescent-phase samples to detect seroconversion or antibody titer rise \(128\). Thus, molecular tests are preferred, depending on the availability of the required samples.

### 5.2 Oropouche virus infection

Oropouche virus (OROV), which causes Oropouche fever, belongs to the Simbu serogroup within the *Orthobunyavirus* genus, *Peribunyaviridae* family. This serogroup includes several viruses subdivided into seven complexes and two phylogenetic subclades \(9\). As is the case with other arboviruses causing febrile illnesses, clinical diagnosis of Oropouche fever is difficult. Although leukopenia and elevated transaminases have been observed in some patients, these findings are nonspecific \(10, 29\). Thus, laboratory diagnosis is essential for the confirmation of Oropouche fever cases. One important thing to consider regarding OROV laboratory diagnosis is that this virus possesses a three-segment genome, with segments S, M, and L (see section 1.1.3). The acute phase of Oropouche fever commonly lasts 2 to 7 days \(9, 10, 29\), during which phase, viral identification can be performed by molecular detection or viral isolation. Molecular methods are useful to detect the viral genome in serum and CSF samples \(9, 10, 29, 126\). Most of these methods are based on detection of the S segment, but targeting the M segment is also important to discriminate OROV isolates from OROV reassortant viruses \(9, 132\). Viral isolation can be performed by intracerebral inoculation of suckling mice or by inoculation into Vero and C6/36 cell monolayers \(9\).

Antibodies are detected in serum during the convalescence phase, usually as of day five of the onset of symptoms \(9, 29\). Serologic diagnosis of OROV disease includes in-house methods, such as PRNT, complement fixation test, immunofluorescence, hemagglutination inhibition tests, and IgM and IgG ELISA \(9, 10, 29\). IgM and IgG antibodies have also been detected in CSF samples \(10\).

As with other arboviruses, molecular tests are preferred, given the availability of the required samples.
Laboratory diagnosis of other neurotropic arboviral infections

Infection by arboviruses from diverse families and from different geographical regions may lead to immediate or delayed neurologic manifestations in humans. These include both flaviviruses (WNV, SLEV, Japanese encephalitis virus [JEV, which does not currently circulate in the Americas], among others) and alphaviruses (e.g., EEEV, VEEV, WEEV). Moreover, DENV, CHIKV, and ZIKV have also been associated with encephalitis and other neurological syndromes (3–5, 55). PAHO and CDC guidelines for laboratory diagnosis of WNV infection (133, 134) and WHO guidelines for JEV (135) are available.

Sera and/or CSF received by the laboratory are initially tested for virus or nucleic acid if the post-symptom onset collection date is within the recommended time frame (Figure 6). For most of these viruses, viremia is very short after the onset of encephalitis. If test results are negative, serologic assays are used for arboviruses selected based on circulating species in the potential geographical area of infection, and whose symptoms are compatible with the case. Neurological infection is confirmed if endpoint RT-PCR and/or real time RT-PCR or virus isolation in CSF samples are positive.

6.1 Virological methods

Neurologic arboviruses are generally present in the blood during the acute phase of infection, i.e., the first 2 to 4 days. As for other arboviruses, the following virological methods can be used.
6.1.1 Molecular detection of viral RNA

RT-PCR on serum or CSF can be used following protocols for real-time or end-point testing and virus-specific primers/probe (see Table 7 for reference assays). Generic protocols (pan-alphavirus and pan-flavivirus) followed by nucleotide sequencing can also be used for virus identification. If testing autopsy samples, such as brain (most common), nucleic acid testing should be done on several tissue sections from different locations in the organ.

6.1.2 Virus isolation

Virus isolation is accomplished by inoculation of blood or sera of the patient in cultured cell lines, such as Vero cells, C6/36 mosquito cells, or alternatively for alphaviruses, duck or chicken embryo fibroblasts. Cell cultures are read on a daily basis under the microscope for evidence of cytopathology. Mice or hamsters 1 to 4 days old can be inoculated intracerebrally if isolating the virus is important and cell culture has not been successful. Isolate identification can be confirmed broadly by immunofluorescence, neutralization, or specifically by RT-PCR or nucleic acid sequence analysis. Immunofluorescence and RT-PCR are fast and provide results within a few hours. Virus isolation can be slow and require multiple
TABLE 7  
Reference molecular assays for the detection of ribonucleic acid from selected neurotropic arboviruses

<table>
<thead>
<tr>
<th>VIRUS</th>
<th>TYPE OF ASSAY</th>
<th>TARGET SEQUENCE</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEEV1,2</td>
<td>Real-time singleplex RT-PCR</td>
<td>E2</td>
<td>Primers/probe: EEE 9391 / EEE 9459c / EEE 9414probes</td>
</tr>
<tr>
<td></td>
<td>Real-time duplex RT-PCR</td>
<td>E1</td>
<td>Duplex with SLEV</td>
</tr>
<tr>
<td>VEEV3</td>
<td>Real-time singleplex RT-PCR</td>
<td>NSP2</td>
<td></td>
</tr>
<tr>
<td>WEEV1,3</td>
<td>Real-time singleplex RT-PCR</td>
<td>E1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>C</td>
<td></td>
</tr>
<tr>
<td>SLEV2,4</td>
<td>Real-time singleplex RT-PCR</td>
<td>prM/E</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Real-time duplex RT-PCR</td>
<td>NS5</td>
<td>Duplex with EEEV</td>
</tr>
<tr>
<td>WNV5</td>
<td>Real-time singleplex RT-PCR</td>
<td>3' UTR</td>
<td>Primers/probe: WN3'NC-forward / WN3'NC-reverse / WN3'NC-probe</td>
</tr>
<tr>
<td></td>
<td></td>
<td>E</td>
<td>Primers/probe: WNENV-forward / WN3'ENV-reverse / WNENV-probe</td>
</tr>
</tbody>
</table>

Note: C: capsid protein; E: envelope protein; EEEV: eastern equine encephalitis virus; NS: nonstructural; prM: premembrane protein; RT-PCR: reverse transcription polymerase chain reaction; SLEV: St. Louis encephalitis virus; UTR: untranslated region; VEEV: Venezuelan equine encephalitis virus; WEEV: western equine encephalitis virus; WNV: West Nile virus.

Sources:  

passages of the culture, with results taking weeks. Sensitivity depends on the condition of the specimen and the cells used.

6.1.3 Immunohistochemistry

Immunohistochemistry or RT-PCR can be performed on paraffin-embedded formalin-fixed tissue samples.

Negative cell culture or tissue test results do not rule out the presence of an infectious agent. The sample may not have been maintained properly to preserve the infectious virus, or the tissue section may be from a location without viral antigen, which might cause negative results. Infections can be focal or of low titers, and, as a result, not detectable.

6.2 Serological methods

Serology is the basis of diagnostics for arboviral infections associated with neurological involvement, since viremia is brief. Serological assays may be conducted on serum and/or CSF. It is recommended to study both samples simultaneously in patients with neurological involvement (See section 4.2.1).
However, collecting CSF is a more invasive procedure, and its volume is usually less than serum, therefore, the latter is used more often in non-neurologic cases.

The available serological methods are described below.

**6.2.1 Detection of immunoglobulin M antibodies**
Detection of virus-specific IgM antibodies in a single serum or CSF sample using IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) supports recent virus infection, but interpretation may be complicated by cross-reaction with other viruses of the same genus, particularly in the case of flaviviruses. Flavivirus-specific IgM antibodies are usually detectable in acute phase serum samples 3 to 8 days after onset of illness, and persist for 30 to 90 days; longer persistence, up to over 1 year, has been documented for WNV and SLEV (136, 137). Therefore, positive IgM antibody results must be interpreted with caution, as they may not represent the current etiologic agent. If serum is collected within 8 days of illness onset, the absence of detectable virus-specific IgM does not rule out the diagnosis of viral infection, and the test may need to be repeated on a later post-onset sample.

Available IgM assays include MAC-ELISAs developed by CDC (EEEV [138]; SLEV and WNV [139]; JEV [140]). For WNV, a number of commercial IgM ELISA assays have been approved by the FDA (99) and for JEV, a commercial IgM ELISA assay has been evaluated against the CDC MAC-ELISA (141). Additionally, a duplex microsphere immunoassay for WNV and SLEV IgM antibody detection has been developed by CDC (142).

**6.2.2 Detection of immunoglobulin G antibodies**
IgG antibodies appear 5 to 7 days after symptom onset. IgG ELISA or microsphere immunoassay can be used to measure IgG antibody levels. A fourfold or greater rise in IgG titer in two serum samples collected, initially, 5 to 7 days after symptom onset, then after at least 3 weeks, indicates infection with the agent against which the sample was tested. Exceptions to this include increased antibody titers due to original antigenic sin, i.e., increase in antibody titer to the primary infecting agent following a secondary infection with a closely related virus (111). A single IgG positive serum sample is only indicative of past infection at an undetermined time.

**6.2.3 Plaque reduction neutralization test**
PRNT in primary infections is very specific. However, in patients with secondary flavivirus infections, virus-specificity is lost due to cross-reactivity between closely related viruses, as well as due to original antigenic sin. Availability of paired serum samples collected in the acute and convalescent phases may be useful for test interpretation but may not for the reasons stated above.

**6.2.4 Immunofluorescent antibodies assays**
These assays detect IgG, IgM, and IgA antibodies in serum. They are broadly reactive but can be used to narrow down identification of a viral agent to the level of viral family. Slides are spotted with infected
Vero cell culture, and an indirect IFA is generally performed using monoclonal antibody for greater specificity, where possible.

Complement fixation and hemagglutination inhibition may also be used, but they have been replaced in most diagnostic laboratories with the assays described above.

6.2.5 Use of chimeric viruses for plaque reduction neutralization tests

PRNT requires using live infectious viruses that plaque well in cell culture. Furthermore, JEV, WNV, and SLEV require biosafety level 3 (BSL-3) containment, not available to many public health laboratories. Dengue viruses 1, 2, 3, and 4 do not require high containment, but also do not always form clear, easy to count plaques, making the PRNT more difficult to read. Similarly, WNV and SLEV may form heterogeneous size plaques that appear at different times. For these reasons, CDC took advantage of ChimeriVax®, an attenuated recombinant virus constructed from YFV vaccine strain 17D, where the chimeric viruses had the prM and E proteins of the virus replaced with those of WNV, SLEV, JEV, or DENV 1–4. These chimeric viruses perform in the PRNT in basically the same way as the wildtype and plaque earlier and better than wildtype DENV 1, 3, 4 (143). Chimeric viruses containing ZIKV prM and E proteins have also been developed for PRNT, yielding plaques 2 days earlier than the wildtype virus.
RNA arboviruses, as most RNA viruses, have high genetic plasticity, with high evolutionary and adaptive potential. Sometimes, there are different types/serotypes within a species, as in the case of DENV (types 1 to 4). Viruses or virus types are subdivided into genotypes, and genotypes into lineages. The viral types, genotypes, and lineages present different genetic characteristics (genotype) that may be reflected in different biological properties (phenotype). Genetic differences among types, genotypes and/or lineages may influence replication in vectors or hosts, viral transmission, and disease severity, and may be associated with viruses’ geographical distribution and epidemic potential. On the other hand, different genetic and biological characteristics exhibited by these viruses may influence diagnostic tests and detection of arboviruses, as in molecular or serological tests.

Genomic surveillance through nucleic acid sequencing reveals the viral types, genotypes and lineages circulating in an area. Phylogenetic and evolutionary analyses of viral nucleotide sequences yield important knowledge and understanding of the origins, evolution, emergence, epidemiology, transmission, and pathogenesis of arboviruses. Such knowledge also supports the establishment of diagnostic, prevention, and control measures. Genotyping of viruses is especially important in the context of new outbreaks, when viruses emerge or reemerge in a specific area, and when atypical clinical outcomes are reported. Nucleic acid sequencing is also used for identifying infecting viruses in positive samples using generic molecular assays (see section 4.1.1.6). Timely genomic surveillance is also
important to monitor the match between sequences of primers and probes used for RT-PCR, and the sequences of the viruses circulating in the Region.

Different nucleic acid sequencing technologies can be used for virus genetic characterization, ranging from Sanger sequencing (incorporation of chain-terminating dideoxynucleotide method) to next-generation sequencing (NGS). Different strategies to amplify and sequence amplicons, or to directly sequence viral genomes without prior amplification, are available (27, 144–146). Using viral nucleotide sequences for phylogenetic analyses one can compare different sequences and determine the viral types and genotypes. Phylogenetic analyses are usually performed with whole genome sequences or sequences of variable genes, such as those encoding envelope proteins which are in general more variable than other structural genes and nonstructural genes. Different strategies can be used for phylogenetic analyses; automated tools for genotyping arboviruses are available online, e.g., for DENV, CHIKV, ZIKV and YFV (147).
References


Human infection with arboviruses, such as dengue, yellow fever, chikungunya and Zika, occurs through the bite of blood-sucking arthropods (mosquitoes, ticks and midges, among others). There are more than 100 types of arboviruses and the infections they trigger range in severity from asymptomatic to life-threatening. In addition, constant evolution of many of these viruses have generated different genotypes and viral variants that might eventually impact the public health systems. Outbreaks caused by these viruses are increasingly common throughout tropical and sub-tropical regions, including much of the Americas, making them an issue of international concern. According to the World Health Organization, almost 4 billion people live in areas where arboviruses are current public health threats.

This publication provides technical recommendations for public health laboratories involved in arbovirus diagnosis and surveillance. It covers the types of specimens most useful for diagnosing infections; lists minimum clinical datasets for sample labeling and analysis; and describes best practices for processing and handling of samples, along with recommended workflows and biosafety considerations. The most appropriate tests for each of the most common arboviruses are presented with recommended diagnostic algorithms. Both direct diagnostic methods – through genome detection, antigen detection and viral isolation – and indirect serological methods (which detect the immune response to viral infection rather than the virus itself) are included.

In addition to its primary target audience of public health laboratory staff, this publication will also be useful to research centers that identify arboviral infections as part of their research.