



# Laboratory Diagnosis

**L**ABORATORY tests essential for confirmatory diagnosis of dengue infection include: (a) isolation of the virus, (b) demonstration of a rising titre of specific serum dengue antibodies, and (c) demonstration of a specific viral antigen or RNA in the tissue or serum<sup>(21, 22)</sup>. Isolation of the virus is the most definitive approach, but the techniques presently available require a relatively high level of technical skill and equipment. Serological tests are simpler and more rapid, but cross-reactions between antibodies to dengue and other flaviviruses may give false positive results. In addition, accurate identification of the infecting dengue

virus serotype is not possible with most serological methods. New technologies available for the laboratory diagnosis of dengue infection include immunohistochemistry on autopsy tissues and polymerase chain reaction (PCR) to detect viral RNA in the tissue or serum<sup>(22)</sup>.

## 5.1 Collection of Specimens

An essential aspect of the laboratory diagnosis of dengue is proper collection, processing, storage and shipment of specimens. The types of specimens and their storage and shipment requirements are presented in Table 4.

Table 4. Collecting and processing specimens for laboratory diagnosis of dengue

Specimen Type	Time of collection	Clot retraction	Storage	Shipment
Acute phase blood (S1)	0-5 days after onset	2-6 hours, 4°C	Serum - 70°C	Dry ice
Convalescent phase blood (S2+S3)	14-21 days after onset	2-24 hours, ambient	Serum - 20°C	Frozen or ambient
Tissue	As soon as possible after death		70°C or in formalin	Dry ice or ambient

Source: Gubler DJ, and Sather GE. 1988<sup>(21)</sup>

- Collect a specimen as soon as possible after the onset of illness, hospital admission or attendance at a clinic (this is called the acute serum, S1).
- Collect a specimen shortly before discharge from the hospital or, in the event of a fatality, at the time of death (convalescent serum, S2).
- Collect a third specimen, in the event hospital discharge occurs within 1-2 days of the subsidence of fever, 7-21 days after the acute serum was drawn (late convalescent serum, S3).

The optimal interval between the acute (S1) and the convalescent (S2 or S3) serum is 10 days. The above recommendations allow for the collection of at least two serum samples for comparison, and ideally will provide for an adequate interval between sera. Serological diagnoses are predicated on the identification of changes in antibody levels over time. Serial (paired) specimens are required to confirm or refute a diagnosis of acute flavivirus or dengue infection.

- The type of specimens to be collected, the way they should be processed for a laboratory diagnosis of dengue, and the information required are presented in this chapter. Effective laboratory support for proactive DF/DHF surveillance requires close and frequent communication between staff in the laboratory and those in the epidemiology unit of the ministry of health. It also requires, at a minimum, weekly evaluation of laboratory results, including monitoring the geographic location of positive cases, the seropositivity rate, the virus serotypes isolated,

and the occurrence of severe and fatal disease. This information must be communicated on a weekly basis to the epidemiology unit for dissemination to other offices in the ministry of health and for further action. Weekly laboratory results are clearly the driving force which determine the response to be taken.

- The above data obtained from a proactive surveillance system can be used effectively if they are disseminated to the proper government and community agencies. Thus, an effective communication or reporting system is also a critical component of the surveillance system. The availability of inexpensive yet powerful desktop computers that are networked can revolutionize surveillance reporting since, with the touch of a button, all responsible persons/agencies can be informed of the latest data needed for decision making.
- Samples of suitable request and reporting forms for arbovirus laboratory examination are provided in Annex II. Blood is preferably collected in tubes or vials, but filter paper may be used if this is the only option. Filter-paper samples cannot be used for virus isolation.

#### **Blood collection in tubes or vials**

- Aseptically collect 2-10 ml of venous blood.
- Use adhesive tape marked with pencil, indelible ink, or a typewritten self-adhesive label to identify the container. The name of the patient, identification number and date of collection must be indicated on the label.

- Use vacuum tubes or vials with screw caps, if possible. Fix the cap with adhesive tape, wax or other sealing material to prevent leakage during transport.
- Ship specimens to the laboratory on wet ice (blood) or dry ice (serum) as soon as possible. Do not freeze whole blood, as haemolysis may interfere with serology test results.
- If there will be more than a 24-hour delay before specimens can be submitted to the laboratory, the serum should be separated from the red blood cells and stored frozen.

#### Blood collection on filter paper

- With a pencil, write the patient's initials or number on two or three filter-paper discs or strips of standardized absorbent paper.\*
- Collect sufficient finger-tip blood (or venous blood in a syringe) on the filter paper to fully saturate it through to the reverse side. Most standard filter-paper discs or strips will absorb 0.1 ml of serum.
- Allow the discs or strips to dry in a place that is protected from direct sunlight and insects. Preferably, the blood-soaked papers should be placed in a stand which allows aeration of both sides. For unusually thick papers, a drying chamber may be useful, e.g. desiccator jar, air-conditioned room, or warm-air incubator.
- Place the dried strips in plastic bags and staple them to the laboratory examination request form. Store without refrigeration.

Dried filter-paper discs may be sent through the mail.

One of the recommended methods for eluting the blood from filter-paper discs and preparing it for the HI or IgM and IgG tests is as follows :

- Elute the disc at room temperature for 60 minutes or at 4°C overnight, in 1 ml of kaolin in borate saline (125 g/litre), pH 9.0, in a test-tube.
- After elution, keep the tube at room temperature for 20 minutes, shaking periodically.
- Centrifuge for 30 minutes at 600g.
- For HI tests using goose erythrocytes, without removing the kaolin, add 0.05 ml of 50% suspension of goose cells to the tube, shake without disturbing the pellet, and incubate at 37°C for 30 minutes.
- Add 1 ml of borate saline, pH 9.0, to the tube.
- Centrifuge at 600g for 10 minutes and decant the supernatant.
- This is equivalent to a 1:30 serum dilution.
- Each laboratory must standardize the filter-paper technique against results with venous blood from a panel of individuals.

## 5.2 Isolation of Dengue Virus

Isolation of most strains of dengue virus from clinical specimens can be accomplished in a majority of cases provided the sample is taken in the first few days of illness and processed without delay. Specimens that may be suitable

\* Whatman No.3 filter-paper discs 12.7 mm (1/2 inch) in diameter are suitable for this purpose, or Nobuto Type 1 blood-sampling paper made by Toyo Roshi Kaisha Ltd., Tokyo, Japan.

for virus isolation include acute phase serum, plasma or washed buffy coat from the patient, autopsy tissues from fatal cases, especially liver, spleen, lymph nodes and thymus, and mosquitoes collected in nature.

For short periods of storage (up to 48 hours), specimens to be used for virus isolation can be kept at +4 to +8°C. For longer storage, the serum should be separated and frozen at -70°C, and maintained at such so that thawing does not occur. If isolation from leucocytes is to be attempted, heparinized blood samples should be delivered to the laboratory within a few hours. Whenever possible, original material (viraemic serum or infected mosquito pools) as well as laboratory-passaged materials should be preserved for future study.

Tissues and pooled mosquitoes are triturated or sonicated prior to inoculation. The different methods of inoculation and the methods of confirming the presence of dengue virus are shown in Table 5.<sup>(22)</sup>

The choice of methods for isolation and identification of dengue virus will depend on

local availability of mosquitoes, cell culture, and laboratory capability. Inoculation of serum or plasma into mosquitoes is the most sensitive method of virus isolation, but mosquito cell culture is the most cost-effective method for routine virologic surveillance. It is essential for health workers interested in making a diagnosis by means of virus isolation to make contact with the appropriate virology laboratory prior to the collection of specimens. The acquisition, storage and shipment of the samples can then be organized to have the best chance of successful isolation.

In order to identify the different dengue virus serotypes, mosquito head squashes and slides of infected cell cultures are examined by indirect immunofluorescence using serotype-specific monoclonal antibodies.

### 5.3 Serological Tests for the Diagnosis of DF/DHF

Five basic serologic tests are routinely used for the diagnosis of dengue infection<sup>(21,23)</sup> haemagglutination-inhibition (HI), complement

Table 5. Dengue virus isolation methods

Recommended methods	Confirmation of dengue virus infection
Inoculation of mosquitoes	Presence of antigen in head squashes demonstrated by immunofluorescence
Inoculation of insect cells or (a) mammalian cultures	Presence of antigen in cells demonstrated by immunofluorescence
	(b) Cytopathic effect and plaque

fixation (CF), neutralization test (NT), IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA), and indirect IgG ELISA. Regardless of the test used, unequivocal serologic confirmation depends upon a significant (4-fold or greater) rise in specific antibodies between acute-phase and convalescent-phase serum samples. The antigen battery for most of these serologic tests should include all four dengue serotypes, another flavivirus, such as Japanese encephalitis, a non-flavivirus such as chikungunya, and an uninfected tissue control antigen, when possible.

#### Haemagglutination inhibition (HI) test

Of the above tests, HI has been the most frequently used for routine serologic diagnosis of dengue infections. It is sensitive, easy to perform, requires only minimal equipment, and is very reliable if properly done. Because HI antibodies persist for long periods (up to 50 years or longer), the test is ideal for seroepidemiologic studies. The HI test is based on the fact that the dengue viruses, under controlled conditions of pH and temperature, can agglutinate goose red blood cells, and this effect can be inhibited by specific antibodies. The antigens employed are prepared from infected suckling mice brains by extraction with sucrose and acetone to remove the lipids, or from infected mosquito cell cultures that have been concentrated or purified. Serum specimens must be treated to remove non-specific inhibitors and agglutinins.

The HI antibody usually begins to appear at detectable levels (titer of 10) by day five or

six of illness, and antibody titers in convalescent-phase serum specimens are generally at or below 1:640 in primary infections, although there are exceptions. By contrast, there is an immediate anamnestic response in secondary and tertiary dengue infections, and antibody titers increase rapidly during the first few days of illness, often reaching 1:5,120 to 1:10,240 or more. Thus, a titer of 1:1,280 or greater in an acute-phase serum is considered a presumptive diagnosis of current dengue infection. High levels of HI antibody may persist for 2-3 months in some patients, but in most antibody titers will generally begin to wane by 30-40 days and fall below the 1:1,280 level.

The major disadvantage of the HI test is lack of specificity, which makes the test unreliable for identifying the infecting virus serotype. However, some primary infections may show a relatively monotypic HI response that generally correlates with the virus isolated<sup>(2)</sup>.

#### Complement fixation (CF) test

The CF test is not widely used for routine dengue diagnostic serology. It is more difficult to perform and requires highly-trained personnel. The CF test is based on the principle that the complement is consumed during antigen-antibody reactions. Two reactions are involved, a test system and an indicator system. Antigens for the CF test are prepared in the same manner as those for the HI test.

CF antibodies generally appear later than HI antibodies, are more specific in primary

infections, and usually persist for shorter periods, although low-level antibodies may persist in some persons. Because of the late appearance of CF antibodies, some patients may show a diagnostic rise by CF, but have only stable antibody titers by HI. The greater specificity of CF test in primary infections is demonstrated by the monotypic CF responses, whereas HI responses are broadly heterotypic. The CF test is not specific in secondary infections. The CF test is useful for patients with current infections, but is of limited value for seroepidemiologic studies where detection of persistent antibodies is important.

#### **Neutralization test (NT)**

The NT is the most specific and sensitive serologic test for dengue viruses. The most common protocol used in most dengue laboratories is the serum dilution plaque reduction neutralization test (PRNT). It is based on the fact that dengue viruses produce cytopathic effects (CPE) which can be observed as plaques in susceptible cell cultures. This CPE is neutralized by the presence of specific antibodies. In general, neutralizing antibodies rise at about the same time or at a slightly slower rate than HI antibodies, but more quickly than CF, and persist for at least 50 years or longer. Because NT is more sensitive, neutralizing antibodies may be detectable in the absence of detectable HI antibodies in some persons with past dengue infection.

The NT can be used to identify the infecting virus in primary dengue infections,

provided the serum samples are properly timed. Relatively monotypic responses are observed in properly timed convalescent-phase serum. As noted above, the HI and CF tests may also give monotypic responses to dengue infection that generally agree with NT results. In those cases where the responses are monotypic, the interpretation is generally reliable. In secondary and tertiary infections, it is not possible to reliably determine the infecting virus serotype by NT. Because of the long persistence of neutralizing antibodies, the test may also be used for seroepidemiologic studies. The major disadvantages are the expense, time required to perform the test, and technical difficulty. It is therefore not routinely used in most laboratories.

#### **IgM-capture enzyme-linked immunosorbent assay (MAC-ELISA)**

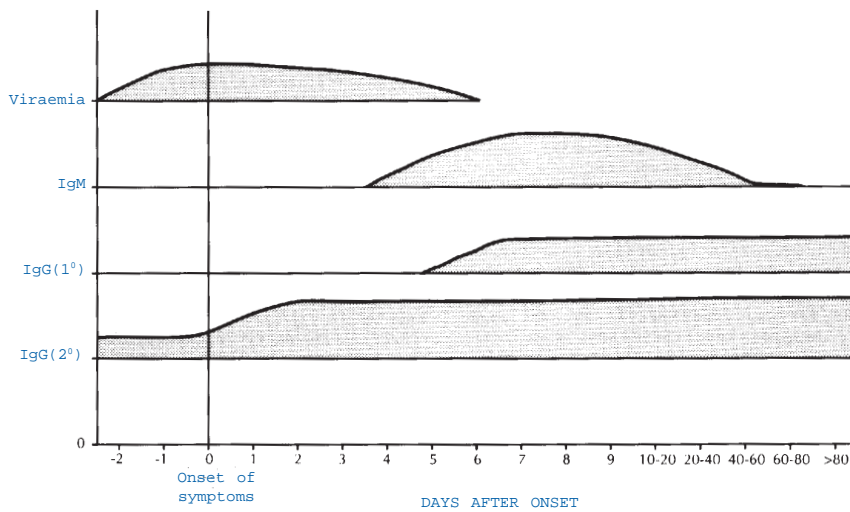
MAC-ELISA has become widely used in the past few years. It is a simple, rapid test that requires very little sophisticated equipment. MAC-ELISA is based on detecting the dengue-specific IgM antibodies in the test serum by capturing them out of solution using anti-human IgM that was previously bound to the solid phase<sup>(24)</sup>. If the IgM antibody from the patient's serum is anti-dengue antibody, it will bind the dengue antigen that is added in the next step and can be detected by subsequent addition of an enzyme labelled anti-dengue antibody, which may be human or monoclonal antibody. An enzyme-substrate is added to give a colour reaction.

The anti-dengue IgM antibody develops a little faster than IgG, and is usually detectable by day five of the illness. However, the rapidity with which IgM develops varies considerably among patients. Some patients have detectable IgM on days two to four after the onset of illness, while others may not develop IgM for seven to eight days after the onset<sup>(22)</sup>. IgM antibody titers in primary infections are significantly higher than in secondary infections, although it is not

uncommon to obtain IgM titers of 320 in the latter cases. In some primary infections, detectable IgM may persist for more than 90 days, but in most patients it wanes to an undetectable level by 60 days<sup>(21)</sup> (Fig.2).

MAC-ELISA is slightly less sensitive than the HI test for diagnosing dengue infection. It has the advantage, however, of frequently requiring only a single, properly timed blood sample. Considering the difficulty in obtaining second blood samples and the long delay

**Figure 2.** Representation of the temporal appearance of virus, IgM, and IgG antibodies in persons infected with dengue virus.



Shaded areas represent approximate time periods when virus or antibody can be detected

in obtaining conclusive results from the HI test, this low error rate would be acceptable in most surveillance systems. It must be emphasized, however, that because of the persistence of IgM antibody, MAC-ELISA positive results on single serum samples are only provisional and do not necessarily mean that the dengue infection is current. It is reasonably certain, however, that the person had a dengue infection sometime in the previous two to three months.

MAC-ELISA has become an invaluable tool for surveillance of DF/DHF/DSS. In areas where dengue is not endemic, it can be used in clinical surveillance for viral illness or for random, population-based serosurveys, with the certainty that any positives detected are recent infections<sup>(21)</sup>. It is especially useful for hospitalized patients, who are generally admitted late in the illness after detectable

IgM is already present in the blood.

### **IgG-ELISA**

An indirect IgG-ELISA has been developed that compares well to the HI test<sup>(23)</sup>. This test can also be used to differentiate primary and secondary dengue infections. The test is simple and easy to perform, and is thus useful for high-volume testing. The IgG-ELISA is very non-specific and exhibits the same broad cross-reactivity among flaviviruses as the HI test; it cannot be used to identify the infecting dengue serotype. However, it has a slightly higher sensitivity than the HI test. It is expected that as more data are accumulated on the IgG ELISA, it will replace the HI test.

### **Rapid serologic test kits**

A number of commercial serologic test kits for anti-dengue IgM and IgG antibodies have become available in the past few years, some producing results within 15 minutes<sup>23</sup>. Unfortunately, the accuracy of most of these tests is unknown since they have not yet been properly validated. Some of the kits that have been independently evaluated at CDC have had a high rate of false positive results compared to standard tests, while others have agreed closely with standard tests. It is anticipated that these test kits can be reformulated to make them more accurate, thus making global laboratory-based surveillance for DF/DHF an obtainable goal in the near future. It is important to note that these kits should not be used in the clinical setting to guide management of DF/DHF cases because many serum samples