CHAPTER 4

Laboratory diagnosis

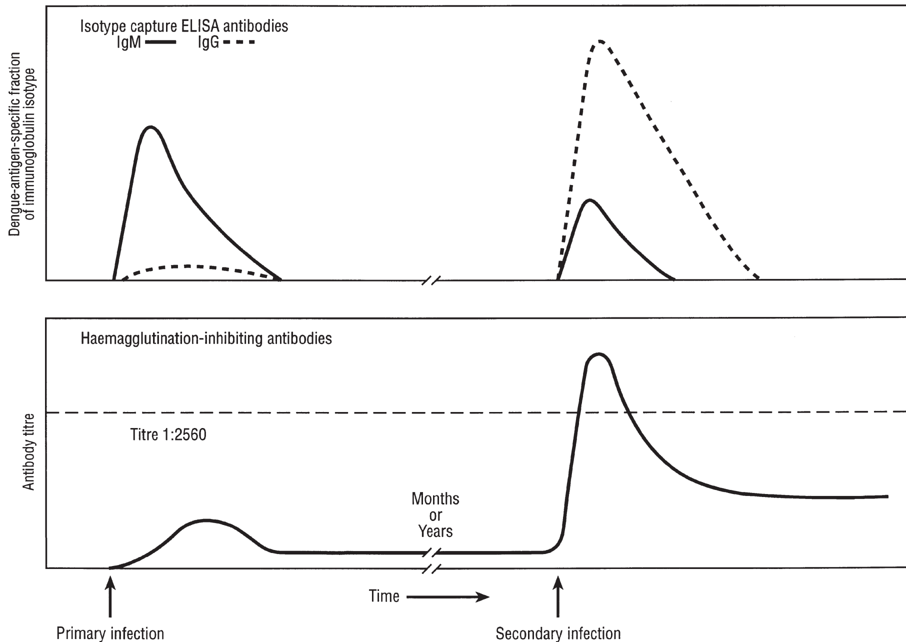
The two basic methods for establishing a laboratory diagnosis of dengue infection are detection of the virus (e.g. culture) or detection of anti-dengue antibodies (serology). Until recently, detection of the virus implied solely the recovery of the virus by culture; however, current procedures can detect dengue virus RNA and specific dengue virus antigens. Consequently, these procedures are likely to become routine as the necessary reagents and instrumentation become more widely available. An understanding of the kinetics of dengue virus replication and host responses, as well as of the collection and handling of specimens, will help clarify the strengths and weaknesses of the two laboratory methods for diagnosing dengue infection.

Kinetics of dengue virus replication and host response

By the time a person infected with dengue virus develops fever, the infection is widely disseminated. The virus is found in serum or plasma, in circulating blood cells and in selected tissues, especially those of the immune system, for approximately 2–7 days, roughly corresponding to the period of fever. Dengue virus usually infects the peripheral blood mononuclear cells within a few days of the infective mosquito bite, and the infection rate revealed by antigen staining is usually 1–10 infected cells per 10 000 cells. Detectable levels of anti-dengue antibodies appear after several days of fever. Two patterns of immune response are distinguished: primary and secondary (anamnestic) (see Figure 4.1).

Persons never previously infected with a flavivirus, nor immunized with a flavivirus vaccine (e.g. yellow fever, Japanese encephalitis, tick-borne encephalitis), mount a primary antibody response when infected with dengue virus. The dominant immunoglobulin isotype is IgM. Anti-dengue IgM detectable by IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) appears in half of the patients with a primary infection while they are still febrile; in the other half, it appears within 2–3 days of defervescence. In one series of dengue patients (infection confirmed by virus isolation or paired serum serology), 80% had detectable levels of IgM antibody by day 5 of illness, and 99% by day 10. Once detectable, IgM levels rise quickly and appear to peak about 2 weeks after the onset of symptoms; they then decline to undetectable

Fig. 4.1
Primary and secondary immunological response in dengue virus infection



levels over 2–3 months. Anti-dengue antibodies inhibit the haemagglutination of gander red blood cells by dengue virus; haemagglutination-inhibiting antibodies appear simultaneously with the detection of IgM by enzyme immunoassay. Anti-dengue IgG appears shortly afterwards. The physiological definition of a primary infection is therefore one characterized by a high molar fraction of anti-dengue IgM and a low molar fraction of anti-dengue IgG.

In primary infection with dengue virus, serological tests may yield results that indicate a specific dengue serotype with specimens obtained early in the disease. In other cases, cross-reactive antibodies, often apparent in the first 1–2 months after infection, may confound determination of the serotype. In such cases, a monotypic antibody specific for the infecting serotype may be detected 3–6 months after infection. Therefore, specimens obtained during late convalescence from patients with a primary seroresponse pattern may be useful in determining the infecting dengue virus serotype.

Individuals with immunity due to previous flavivirus infection or immunization mount a secondary (anamnestic) antibody response when infected with dengue virus. In secondary flavivirus infections, which account for most cases of DHF, the dominant immunoglobulin isotype is IgG. Anti-dengue IgM

appears in most instances, and while the kinetics of IgM production are similar to those observed in primary infections, the levels of IgM are dramatically lower. In contrast to primary infection, secondary infection with dengue virus results in the appearance of high levels of anti-dengue IgG before, or simultaneously with, the IgM response. Once detected, IgG levels rise quickly, peak about 2 weeks after the onset of symptoms and then decline slowly over 3–6 months. Anti-dengue IgM levels also peak at about 2 weeks, begin to wane thereafter and are detectable in about 30% of patients 2 months after the onset of symptoms. The physiological definition of a secondary infection is one characterized by a low molar fraction of anti-dengue IgM and a high molar fraction of IgG that is broadly reactive to flaviviruses.

Both IgM and IgG anti-dengue antibodies neutralize dengue virus. The neutralizing antibodies rapidly increase as fever subsides and interfere with the recovery of the virus from serum.

Collection and handling of specimens

When collecting blood specimens from patients with suspected dengue infections, health care workers should:

- 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 should allow the collection of at least two serum samples for comparison, and ideally will provide for an adequate interval between sera. Similar practices would apply to outpatients in clinics. 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.

An abbreviated case history, including the following information, should accompany specimens: the patient's name and registration number, address, age, sex, date of onset of illness, date of hospitalization, attending physician's name, date of the collection of the specimen, and concise clinical findings.

Blood may be collected in tubes or vials or on filter-paper. High-quality absorbent paper has been used for many years to facilitate the shipment of blood specimens to central laboratories for serology. Blood or specimens may also be mailed to a laboratory in sterile, plastic specimen vials or tubes, in accordance with pertinent postal regulations. In the absence of microbial

contamination, exposure to ambient temperatures for up to 7 days while in transit will not significantly alter the results of standard dengue serology tests. Many laboratories now prefer to receive blood specimens in vials or tubes rather than blotted on paper, since the latter require special pretest processing. Annex 5 shows a sample request form for laboratory examination and an arbovirus laboratory reporting form, for use with filter-paper discs. If tubes or vials are used, similar information should be provided.

Specimen-collection procedures: tubes or vials

- Aseptically collect 2–5 ml or more of venous blood.
- Use adhesive tape marked with pencil or indelible ink or a typewritten or printed self-adhesive label to identify the container. At a minimum, the name of the patient, the identification number and the date of collection should be indicated.
- Use tubes or vials with screw-caps, if possible. Fix the cap with adhesive tape, wax or other sealing material to prevent leakage during transport.
- If a specimen cannot be analysed or shipped within 24 hours of being drawn, the serum should be separated from the cells and stored frozen.
- Ship specimens for culture or serology on wet ice to a laboratory as soon as possible.

Do not send frozen whole blood, as the resulting haemolysis can interfere with some tests. Specimens intended only for serology may tolerate shipment at ambient temperatures, particularly if an antiseptic is added to each specimen (e.g. sodium azide, thiomersal).

Specimen-collection procedures: filter-paper

- With a pencil, write the patient's initials or number on 2 or 3 discs or strips of standardized absorbent paper.
- Collect sufficient finger-tip blood (or venous blood in a syringe) on the filter-paper to saturate it through to the reverse side.
- Allow the discs or strips to dry in a place protected from direct sunlight and insects. Preferably, the blood-soaked papers should be placed in a stand that allows aeration of both sides. For unusually thick paper, a drying chamber may be useful, e.g. desiccator jar, air-conditioned room, warm-air incubator.
- Place the dried strips in plastic bags and staple them to corresponding laboratory examination request forms. Once dried, the plastic-enclosed strips may be stored at ambient temperature and mailed to the laboratory.

A question frequently posed concerns the minimum volume of serum required for diagnostic tests. Most assays require 0.1 ml of undiluted specimen. Adequate specimen volume, i.e. 0.3 ml to 2.0 ml of serum, should be submitted

to permit tests in several assays or to allow repeated testing if necessary. Filter-papers with an absorbency equivalent to Whatman No. 3 or Nobuto Type 1 should be used.[1]

Handling specimens for virus culture

Because dengue virus is heat-labile, specimens awaiting transport to the laboratory, including anticoagulated whole blood for the culture of leukocytes, should be kept in a refrigerator or packed in wet ice. For storage up to 24 hours, specimens to be used for virus isolation are preferably kept at +4 °C to +8 °C; for longer storage, serum and tissue specimens should be frozen at −70 °C. In the latter case, they should be so maintained as to prevent thawing. If specimens are frozen with dry ice, they should be placed in a gas-impermeable secondary container (e.g. a heat-sealed plastic bag), since a low pH will inactivate dengue viruses. These storage conditions are to be considered optimal; failure to adhere to them does not result in the complete inactivation of dengue virus in specimens. Dengue virus has been recovered from clinical specimens packed in wet ice or maintained at ambient temperatures for several days.

Diagnostic approach: virus detection versus serology

Detection of dengue virus by culture is the definitive diagnostic test, but practical considerations limit its use. Most importantly, the period when dengue virus can be successfully detected is brief. Within a day or two after the subsidence of fever, rising levels of antibody interfere with virus culture. Furthermore, as noted above, dengue virus is generally heat-labile and special precautions must be taken against the thermal inactivation of specimens. Lastly, since laboratories equipped and staffed to culture viruses are expensive to develop and maintain, their services are not widely available.

Detection of dengue RNA using specific oligonucleotide primers, reverse transcriptase and thermostable polymerase—a test known as the reverse transcription–polymerase chain reaction (PCR) amplification assay—has been successfully employed in several laboratories. The test uses RNA extracted from serum, plasma or cells. Although detection of dengue RNA by this technique is no less complex or expensive than virus culture, it is faster. Without proper precautions, however, contamination can lead to false-positive results. Nevertheless the procedure may be amenable to commercialization as a kit. More-

[1] To prepare blood samples on filter-paper for ELISA assay (IgG or IgM):

- Elute discs in 1.0 ml of phosphate-buffered saline for 60 minutes at room temperature, or overnight at 4 °C.
- Remove discs and centrifuge at 600*g* for 15 minutes to remove any particulate matter.

This results in approximately a 1 : 20 final serum dilution. Each laboratory must standardize the filter-paper technique against results with a panel of venous blood specimens.

over, for technical reasons (discussed below), the test procedure may be particularly useful for detecting viraemia after the subsidence of fever, or in situations where sample handling has not been optimal for virus culture.

Dengue RNA or antigen may also be identified in individual cells using *in situ* hybridization or immunocytochemistry. The former method is theoretically more sensitive, but few laboratories have experience in applying the technique to the detection of dengue RNA in infected tissues. In contrast, antigen detection by immunocytochemistry is simpler, has been employed for a number of years and, with new commercially available reagents, may offer the same specificity as virus isolation.

Serological diagnosis is unhindered by the limitations of virus culture and other direct detection methods. The timing of specimen collection can be more flexible because anti-dengue antibody responses last for at least several weeks after the onset of illness. Immunoglobulins are not easily inactivated by the harsh treatment of specimens: a serum is usually suitable for testing even after prolonged exposure to tropical temperatures. The services of serology laboratories are also more readily available, the assay techniques are relatively simple and some reagents are commercially available.

On the other hand, serological tests may produce false-positive results, which may be due to polyclonal B-cell activation or cross-reactive antibodies elicited by certain group, complex and subcomplex epitopes common to flaviviruses. Shared epitopes, especially those of the envelope glycoprotein (the major structural antigen), cause early antibodies elicited by infection with one dengue serotype to cross-react with other serotypes. Moreover, antibodies elicited by other flaviviruses (e.g. Japanese encephalitis virus) may cross-react with dengue virus. Another factor confounding flavivirus serology is a physiological principle termed “original antigenic sin”, i.e. many B-cell clones responding to a first flavivirus infection will be restimulated to synthesize early antibody with a greater affinity for the first infecting virus than for the current infecting virus in every subsequent flavivirus infection. False-positive reactions can confound the diagnosis of dengue, especially in areas where other flaviviruses are present. By specifically detecting anti-dengue IgM responses, more accurate diagnosis of flavivirus infections may be achieved, although this may not allow determination of the specific dengue serotype causing the infection.

In summary, serological diagnosis is in general less specific than diagnosis by culture. The serological diagnosis of dengue in populations exposed to additional flaviviruses is very challenging—some degree of uncertainty is inevitable. Greater confidence in serological diagnosis may be gained by using neutralization tests (see below), which are usually able to distinguish between the immune responses in primary infections to different dengue serotypes. All too often, however, the most precise serological diagnosis many laboratories can render is *acute flavivirus infection*, rather than *acute dengue infection*.

The selection of laboratory methods must be tailored to meet the objectives of the clinician and the constraints of the clinical specimens available. More-

Table 4.1
Suitable specimens for culture

Source	Material
Patient	Serum, plasma, leukocytes washed to remove antibody, cerebrospinal fluid[a]
Autopsy	Homogenized or minced tissues, e.g. liver, lung, spleen, lymph nodes, thymus; cerebrospinal fluid, pleural fluid, serum, plasma
Vector mosquito	Homogenized pooled mosquitos

[a] Where clinically indicated.

over, the certainty and speed of diagnosis must be balanced against the cost and availability of the tests. Determinations of both the virus *and* the antibody type are preferable to either approach alone. Moreover, the use of alternative methods to confirm or refute test results—e.g. MAC-ELISA and the haemagglutination-inhibition (HI) test—further improves the quality of a laboratory diagnosis.

Laboratory safety precautions

The collection and processing of blood and other specimens places health care workers, particularly those in the laboratory, at risk for exposure to potentially infectious materials. To minimize the risk of infection, safe laboratory techniques must be practised.[1] Such techniques include the use of personal protective equipment and appropriate containers for collecting and transporting samples and adherence to proper procedures for the separation of serum, the handling of glass and sharp instruments and the decontamination and disposal of potentially infectious materials.

Technical aspects of available assays

Isolation of virus

Since all patients with dengue virus infection have a period of viraemia, the isolation of dengue virus from clinical specimens is frequently possible. Factors favouring the successful isolation of virus are collection of the specimen early in the course of disease (usually within 5 days after the onset of fever) and proper handling and prompt delivery of the specimen to the laboratory. Suitable specimens for culture are shown in Table 4.1.

Different methods of confirming the presence of dengue virus are given in Table 4.2. The choice of method depends on the local availability of mosqui-

[1] Detailed guidelines are presented in *Laboratory biosafety manual*, 2nd ed. Geneva, World Health Organization, 1993.

Table 4.2
Methods for isolation of dengue virus

Method	Result confirming presence of dengue virus
Inoculation of mosquitos (adults or larvae)	Detection of antigen in head squash by serotype-specific immunofluorescence
Inoculation of various mammalian or insect cell cultures	Detection of antigen by antibody staining Cytopathic effect; identification of virus upon subpassage Plaque formation; identification of virus upon subpassage
Intracranial inoculation of suckling mice	Presence of antigen in brain detected by antibody staining Symptoms or signs indicating encephalitis Identification of virus upon subpassage

tos, cell cultures and mice. Precautions must always be taken to prevent the laboratory contamination of specimens.

The inoculation of clinical specimens into adult or larval mosquitos is the most sensitive dengue virus culture technique. Suitable specimens for inoculation include serum, plasma, other normally sterile body fluids (e.g. cerebrospinal fluid, pleural fluid), peripheral blood leukocytes and tissue homogenates. Infection is detected by immunofluorescence of a tissue smear prepared from the crushed head of the mosquito (head squash). Generally, mosquitos in the genus *Toxorhynchites* are used. Their large size facilitates inoculation; moreover, because they are not haematophagous, inoculated mosquitos can be handled safely. Finally, the culture of specimens in mosquitos reduces the risk of laboratory errors due to the cross-contamination of cultures. Nevertheless, because the inoculum volume is small, high-sensitivity culture requires the inoculation of 5–20 mosquitos per specimen. However, the raising of *Toxorhynchites* mosquitos is labour-intensive because the larvae are carnivorous and the insectary must therefore maintain production of a second mosquito species as a food source. Adult *male* mosquitos of *Ae. aegypti* and *Ae. albopictus* species can be inoculated with a sensitivity and safety equal to those obtained with *Toxorhynchites* spp. Although male *Ae. aegypti* and *Ae. albopictus* mosquitos are easier to maintain, they require more delicate inoculation techniques because of their smaller size.

In laboratories where colonized mosquitos are not available, specimens may be inoculated in any of several widely available mosquito cell lines (e.g. C6/36 or AP-61 cells). This approach may be slightly less sensitive than inoculation in live mosquitos, but with a larger possible inoculum volume it has adequate sensitivity for routine virus isolation. Since only some dengue virus isolates induce a cytopathic effect in mosquito cells, cell cultures must be screened for specific evidence of infection by an immunoassay. If inoculated cells can be assayed in their culture vessel, additional efficiency is achieved. Because mos-

quito cell lines may be propagated at ambient tropical temperatures (25–34 °C), it is even possible for mosquito cells to be carried to the bedside or into the field, although a sterile culture environment would still be required. The culture of clinical specimens (e.g. anticoagulated whole blood) at the bedside is a means of virus culture that has yet to be fully exploited.

The culture of specimens in vertebrate cell lines (e.g. VERO, LLC-MK_2) and in intracerebrally inoculated newborn mice are the least sensitive methods. On the other hand, the appearance of plaques in these cells lines, or encephalitis in mice, does constitute presumptive evidence of the presence of an arbovirus.

Once a virus has been isolated in culture, serotype-specific anti-dengue monoclonal antibodies[1] are used to examine mosquito head squashes, infected cells, infected cell-culture fluids, or mouse-brain touch preparations for identification. The binding of a specific monoclonal antibody is revealed with a second labelled antibody. Serotype-specific assays may be supplemented with a flavivirus-group-reactive or dengue-complex-reactive monoclonal antibody to serve as a positive control.

Antigen detection in fixed tissues

Flavivirus antigens may be detected in peripheral blood leukocytes from patients with dengue, especially during the febrile phase of illness. Dengue antigen also may be found in the liver and lung at autopsy, and less often in the thymus, lymph nodes, skin, spleen, bone marrow and serosa. Fluorescent antibody, immunoperoxidase and avidin–biotin enzyme assays have been standardized in a number of research laboratories, permitting the visualization of viral antigen in acetone-fixed leukocytes, snap-frozen tissue and even formalin-fixed tissue after limited protease-digestion (to reveal antigens cross-linked by formalin). Tissues should be collected as soon after death as possible, since a delay of even 24 hours compromises antigen staining.

If facilities for virus isolation are available, the tissues should be divided, with a portion placed in fixative (buffered formalin, if not otherwise specified by the immunohistochemistry laboratory) for antigen staining and a portion placed in ice-cold, sterile, isotonic buffer for virus isolation. When possible, heart blood and other body fluids (cerebrospinal fluid, pleural fluid or ascites) should be collected for culture and IgM detection. In the event of the failure of virus isolation, assay of these fluids for IgM and IgG antibodies with a panel of relevant antigens offers the best opportunity to identify the infecting virus.

[1] Dengue type-specific monoclonal antibodies or hybridomas may be obtained from Division of Vector-borne Infectious Diseases, Centers for Disease Control and Pevention, Fort Collins, CO 80522, USA (fax: (303) 221 6428).

Reverse transcription–PCR amplification of dengue RNA

PCR amplification can be used to amplify RNA if reverse transcription of the target RNA to cDNA is used as the initial step. If dengue-specific oligonucleotide primers are employed, reverse transcription–PCR amplification assays can detect a small number of dengue RNA molecules among millions of other RNA molecules. Unlike more limited biological amplification through culture, million-fold enzymatic amplification can be accomplished in a matter of hours. Moreover, since nucleic acid can be separated from proteins in a specimen-preparation step, dengue RNA can be purified from immune complexes. Thus, reverse transcription–PCR amplification introduces for the first time a technique with the potential to detect dengue virus during convalescence, when circulating antibodies otherwise preclude its detection. These assays demand meticulous technique, however, as they are highly prone to false-positive results due to contamination.

Several laboratories have published reverse transcription–PCR amplification protocols to detect dengue viraemia. These methods feature two strategies for identification of the four dengue serotypes: combination of the four serotype-specific oligonucleotide primer pairs in a single reaction tube, or use of a universal dengue oligonucleotide primer pair, which requires a subsequent step to classify positives with serotype-specific oligonucleotides. Both approaches have proved highly successful in preliminary trials. Nevertheless, reverse transcription–PCR amplification of dengue RNA must still be considered an investigational approach; its fuller application awaits greater experience, consensus on the optimal preparation of specimens and determination of the oligonucleotide primer sequences capable of detecting all or most dengue genotypes in circulation.

Serological tests

Diagnosis of dengue by the recovery of virus or the detection of antigens is preferable to serological diagnosis; however, the latter is used to confirm most dengue infections. Although serological assays can, in many instances, provide a presumptive diagnosis of *recent* infection from a single serum specimen, a conclusive diagnosis of *acute* infection can be made only when rising levels of anti-dengue immunoglobulin are detected in paired sera. The diagnosis of acute dengue infection is possible on this basis because antibody levels are known to rise only for 2–4 weeks following infection. The subsequent decline to baseline levels requires another 6–24 weeks, during which time single serum assays may still reveal elevated anti-dengue IgM or IgG antibody. The most commonly used serological techniques for the diagnosis of dengue infection are MAC-ELISA and the HI test. Descriptions of these and less commonly used serological tests follow.

MAC-ELISA

In primary or secondary dengue infections, MAC-ELISA can measure a rise in dengue-specific IgM, even in sera samples collected at 1-day to 2-day intervals in the acute phase. Specimens collected over an interval of 2–3 days spanning the day of defervescence are also usually diagnostic in MAC-ELISA. In cases where only a single specimen is available, detection of anti-dengue IgM permits the diagnosis of recent dengue infection even in primary infections where the level of HI antibody would not be diagnostic.

Because anti-flavivirus IgM is complex-specific (i.e. IgM elicited by dengue virus can generally be differentiated from IgM elicited by Japanese encephalitis, St Louis encephalitis, Murray Valley encephalitis, West Nile or Kunjin viruses), diagnosis of acute or recent dengue infection can be made by tests against a panel of antigens. This feature assumes importance when evaluating cases where symptoms may be attributable to different viruses (e.g. fever and rash may be caused by infection with dengue virus or West Nile virus; fever, clouded consciousness and neurological deficits may be caused by infection with dengue virus or any virus of the mosquito-borne flavivirus encephalitis complex). It should be emphasized, however, that some flavivirus cross-reactivity occurs,

Table 4.3
Interpretation of MAC-ELISA results[a]

IgM antibody response	S1–S2 interval[b]	IgM to IgG ratio	Interpretation
Increase in molar fraction	2–14 days	High	Acute flavivirus infection, primary
		Low	Acute flavivirus infection, secondary
Elevated, no change or decrease in molar fraction	2–14 days	High	Recent flavivirus infection, primary
		Low	Recent flavivirus infection, secondary
Elevated	(Single specimen)	High	Recent flavivirus infection, primary
		Low	Recent flavivirus infection, probably secondary

[a] Criteria derived empirically from data collected at the U.S. Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Laboratories should assess the sensitivity of their assay with standard sera available from Department of Virology, U.S. Armed Forces Research Institute of Medial Sciences, 315/6 Rajvithi Road, Bangkok 10400, Thailand (fax: 66-2-664-4760), WHO Collaborating Centres for Arboviruses and/or Haemorrhagic Fever Reference and Research, or WHO Collaborating Centres for New, Emerging and Re-emerging Diseases (see Annex 6). To infer that dengue virus elicited anti-flavivirus IgM, laboratories must test with a regionally appropriate panel of flavivirus antigens. Results expressed as the ratio of optical density (OD) (test sample) : OD (positive reference sample) will reduce inter-asay variation. Results greater than 2 standard deviations from the mean of negative control sera are presumed to indicate elevated levels of anti-flavivirus IgG or IgM. Laboratories should determine appropriate criteria for categorizing primary and secondary seroresponses. Laboratories should also assess their assay's specificity with a serum panel lacking flavivirus antibodies.

[b] Guidelines do not apply to intervals between acute (S1) and convalescent (S2) specimens greater than 14 days.

and the results of other serological, virological and epidemiological tests should also be used to determine conclusively the infecting virus.

A further advantage of MAC-ELISA is that it may be used without modification to detect anti-flaviviral IgM in cerebrospinal fluid. Since IgM does not normally cross the blood–brain barrier, detection of IgM in cerebrospinal fluid is a significant diagnostic finding, implying flavivirus replication within the central nervous system.

MAC-ELISA provides more information, is more efficient than other serological tests and is especially valuable for laboratories that perform a high volume of testing. A barrier to its wider use is the lack of standardized reagents. Several versions of the test that are performed in 96-well plates and read with spectrophotometric plate readers have been described. A common feature is the use of commercially available anti-IgM as a coating on the plates in order to capture a random sample of isotype immunoglobulin molecules from the test serum. The serum specimen may be tested at a single or multiple dilution(s) with an excess of dengue antigen prepared from infected mouse brain or cell culture lysates, and the use of a detector antibody to measure the quantity of dengue antigen bound by the test serum. The performance characteristics of individual assays are determined by the type and amount of dengue antigen, the detector antibody and the positive and negative controls used to set a cut-off for test positivity. The interpretation of MAC-ELISA results is summarized in Table 4.3.

Haemagglutination-inhibition test

The HI test is simple, sensitive and reproducible and has the advantage of using reagents that may be prepared locally. A disadvantage is that sera samples must be pretreated with acetone or kaolin, to remove non-specific inhibitors of haemagglutination, and then absorbed with gander or type O human red blood cells, to remove non-specific agglutinins. Furthermore, the optimal use of the HI test requires paired sera. Paired sera are most easily obtained upon hospital admission (acute) and discharge (convalescent); if the interval between the first and second serum is less than 7 days, an HI test may not afford a diagnosis in a primary infection. It also normally fails to discriminate between infections by closely related flaviviruses, e.g. between dengue virus and Japanese encephalitis virus, or dengue and West Nile virus.

Dengue viruses agglutinate gander erythrocytes and those of certain other species as well as trypsinized type O human red blood cells. The HI test is based on the ability of dengue virus antibodies to inhibit this agglutination. The test is described in most virology manuals.

- Sera should be extracted with kaolin or acetone and absorbed with gander or trypsinized type O human red blood cells.
- All sera from a single patient should be tested in the same assay using 4–8

Table 4.4
Interpretation of dengue haemagglutination-inhibition antibody response[a]

Antibody response	S1–S2 interval[b]	Convalescent titre[c]	Interpretation
⩾4-fold rise	⩾7 days	⩽1:1280	Acute flavivirus infection, primary
⩾4-fold rise	Any specimen	⩾1:2560	Acute flavivirus infection, secondary
⩾4-fold rise	<7 days	⩽1:1280	Acute flavivirus infection, either primary or secondary
No change	Any specimen	>1:2560	Recent flavivirus infection, secondary
No change	⩾7 days	⩽1:1280	Not dengue
No change	<7 days	⩽1:1280	Uninterpretable
Unknown	Single specimen	⩽1:1280	Uninterpretable

[a] These criteria were derived empirically from data collected at the U.S. Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand. Laboratories should assess the sensitivity of their assay with standard sera from WHO Collaborating Centres for Arboviruses and/or Haemorrhagic Fever Reference and Research or WHO Collaborating Centres for New, Emerging and Re-emerging Diseases (see Annex 6). Laboratories should also establish baseline data for the population they serve during a period of little or no flavivirus transmission.
[b] Interval in days between acute (S1) and convalescent (S2) specimens.
[c] Against any dengue antigen.

haemagglutinating units of the four dengue antigens. (As a screening test, a single broadly reactive dengue antigen may be used (usually DEN-1 or DEN-4) with only a slight loss of sensitivity for primary infections. If a screening test of paired sera is negative, the specimens may be retested against all dengue antigens.)

- Negative serum pairs should be tested against chikungunya antigen where this virus is known to be endemic.
- Known positive and negative sera should be included in each test to standardize results and maintain quality control; reference sera may be available from WHO Collaborating Centres for Arboviruses and/or Haemorrhagic Fever Reference and Research or WHO Collaborating Centres for New, Emerging and Re-emerging Diseases (see Annex 6).

The response to a primary dengue infection is characterized by the slow evolution of the haemagglutination-inhibiting antibody. Because the HI assay does not differentiate among immunoglobulin isotypes, the identification of a primary antibody response must be inferred from the low level or absence of detectable antibody in the acute-phase serum drawn before day 5, as well as from the levels of antibody titres elicited (Table 4.4). The secondary antibody response to dengue is characterized by the rapid evolution of haemagglutination-inhibiting antibody. All antibodies are broadly flavivirus-reactive so a specific diagnosis is not possible on the basis of this test alone. In positive tests there are fourfold or greater increases in titre between acute and convalescent sera, with peak titres always exceeding 1:1280 in secondary responses, and generally falling below this ratio in primary responses.

Neutralization tests

Although several neutralization tests have been described for dengue virus, the most sensitive and specific method is the serum dilution, virus-constant, plaque-reduction test. Following primary dengue infection, relatively specific neutralizing antibodies are detected in early convalescence. Following secondary dengue infections, high-titre neutralizing antibody is produced against at least two and usually all four dengue virus serotypes, as well as against other flaviviruses. In many combinations of sequential infections when appropriately timed specimens are tested, the highest neutralizing antibody titre in convalescent serum is directed against the virus with which the patient was previously (not most recently) infected.

Dot-blot immunoassay

Dot-blot immunoassay technology is relatively new, and reagents and test procedures are evolving. At least one dot-blot immunoassay for dengue antibodies is available commercially. As greater interest develops among commercial manufacturers, additional dot-blot immunoassays are likely to enter the market.

Complement-fixation test

The complement-fixation test may also be used in serological diagnosis, although it is the least sensitive serological assay, and other assays have generally replaced this method. Complement-fixing antibody typically appears later than IgM or HI antibody and is usually more specific. Therefore, it can be useful in confirming dengue infection in patients with paired serum samples taken late in the infection. A fourfold rise in complement-fixing antibody, where the interval between the acute and convalescent serum is less than 2 weeks, signifies a secondary seroresponse pattern.