Dengue diagnostics: proceedings of an international workshop

4–6 October 2004 | WHO/TDR | Geneva, Switzerland
DENGUE DIAGNOSTICS:

proceedings of a joint
TDR/WHO and PDVI workshop

4–6 October 2004  |  WHO/TDR  |  Geneva, Switzerland
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<tbody>
<tr>
<td>AUC</td>
<td>area-under-the-curve</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<td>DEN</td>
<td>dengue</td>
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<tr>
<td>DENV</td>
<td>dengue virus</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DOR</td>
<td>diagnostic odds ratio</td>
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<tr>
<td>DRD</td>
<td>Diagnostics Research and Development (TDR)</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>HAI</td>
<td>haemagglutination inhibition</td>
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<tr>
<td>ICT</td>
<td>immunochromatographic test</td>
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<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
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<tr>
<td>LACV</td>
<td>La Crosse virus</td>
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<tr>
<td>LR</td>
<td>likelihood ratio</td>
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<tr>
<td>MAC-ELISA</td>
<td>IgM antibody capture ELISA</td>
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<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>NASBA</td>
<td>nucleic acid sequence-based amplification</td>
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<tr>
<td>NPV</td>
<td>negative predictive value</td>
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<tr>
<td>NS1</td>
<td>nonstructural glycoprotein 1</td>
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<td>POCT</td>
<td>point-of-care test</td>
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<td>PPV</td>
<td>positive predictive value</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>ROC</td>
<td>receiver operating characteristic</td>
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<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<td>RT-LAMP</td>
<td>real-time reverse transcription loop-mediated isothermal amplification assay</td>
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<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
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<td>SLEV</td>
<td>St Louis encephalitis virus</td>
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<tr>
<td>SMB</td>
<td>suckling mouse brain</td>
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<tr>
<td>SROC</td>
<td>summary receiver operator characteristic</td>
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<tr>
<td>TDR</td>
<td>UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases</td>
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<tr>
<td>Tp</td>
<td>time of positivity</td>
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<td>PDVI</td>
<td>Pediatric Dengue Vaccine Initiative</td>
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<td>POWV</td>
<td>Powassan virus</td>
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<td>QA</td>
<td>quality assurance</td>
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<td>quality control</td>
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<td>UNDP</td>
<td>United Nations Development Programme</td>
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<td>UNICEF</td>
<td>United Nations Children's Fund</td>
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<td>West Nile virus</td>
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<td>World Health Organization</td>
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Executive summary

INTRODUCTION: THE DIAGNOSTIC CHALLENGE

Dengue is diagnosed by isolation of the virus, by serology, or by molecular diagnostic methods. Although several commercial kits for the diagnosis of dengue are available, concerns have arisen with respect to the performance characteristics of these kits. In theory, it should be possible to establish a diagnosis of on-going or recent dengue infection during the acute or early convalescent stages of the disease. When such tests require the identification of the virus or the viral genome they are expensive and require specialized laboratories. Affordable commercial kits of adequate sensitivity and specificity that are able to diagnose dengue infection during the acute stage have not been developed. It is also possible that virological or physiological tests applied during the acute stage of the illness might be capable of predicting the severity of dengue virus infection.

Objectives
The aims of this workshop were to review available data on the performance and utility of rapid tests, to investigate new developments in dengue diagnostics, and to develop strategies for the selection of the best tests to assist in the diagnosis of the disease.

Specific outcomes
An up-to-date inventory of the current rapid tests was presented and the utility of selected tests was discussed. The current industry activities regarding new approaches to diagnostics for dengue and recommendations for the characteristics of ideal diagnostics for specific indications was provided.

Structure of the workshop
The first part of the workshop comprised a discussion on Laboratory diagnosis of dengue. The sections relevant to Laboratory diagnosis included presentations relating to immunological responses in dengue, an overview of methods for laboratory diagnosis, a focus on patient screening, epidemiology, and virological diagnosis. The laboratory diagnosis of dengue using commercial kits was also discussed. The second day featured Diagnostic needs for dengue and covered specific methods for the diagnosis of dengue by means of the detection of antigen and antibody, including the use of recombinant molecules. The use of molecular diagnostics was also presented. Later sessions were devoted to Strategic planning, with presentations covering the challenges ahead for dengue diagnostics, including the need for quality assurance and a network of reference laboratories.

SUMMARY OF DISCUSSION OF TESTS FOR ACUTE INFECTION

It was generally agreed that acute infection with dengue virus was implicated when dengue virus was isolated or the specific dengue virus genome was identified by reverse transcription-polymerase chain reaction (RT-PCR) from serum or plasma
during an acute febrile illness consistent with a dengue syndrome. Circulating levels of nonstructural glycoprotein 1 (NS1) correlate with titres and increases in levels of specific immunoglobulin M (IgM), evidencing a recent acute infection with dengue virus. Use of the following diagnostic tests were recommended:

**Virus isolation**

Virus isolation by inoculation in cell cultures or mosquitoes followed by detection of specific antigens using indirect immunofluorescence is considered to be the “gold standard,” although it requires technical expertise, and is time consuming and relatively expensive.

**Reverse transcription-polymerase chain reaction (RT-PCR)**

RT–PCR, such as one-step or nested RT–PCR or the isothermal nucleic acid sequence-based amplification (NASBA) for RNA, are now widely used to detect dengue viral genes in acute-phase serum samples. The detection of the dengue genome coincides with the onset of viraemia. RT–PCR is not considered to be a rapid test and the high degree of variability between laboratories suggests that more work is required for standardization to be achieved.

**IgM and IgG enzyme-linked immunosorbent assay (ELISA) in acute primary infections**

Patients with a primary infection produce an IgM response to dengue virus, which starts as early as the third day after the onset of fever, but is not uniformly detected until 7 or more days after the onset of fever. The IgM titre continues to rise for 1 to 3 weeks and is detectable for up to 2 months after infection. IgG antibodies to dengue virus are produced approximately 2 weeks after a primary dengue infection and are maintained for life. An acute primary infection with dengue virus can be identified by IgM:IgG ratios in an IgM capture ELISA with acute- and convalescent-phase specimens taken at an interval of approximately 7 days.

**SUMMARY OF TESTS DISCUSSED FOR SECONDARY INFECTION**

As in primary dengue infections, IgM may be detected as early as 3 days after the onset of fever during a secondary infection, but not optimally until at least 5 days after the onset of fever, while the IgG titre rises rapidly from 3–5 days after the onset of fever. Thus, patterns of IgM and IgG reactivity determined by ELISA in sera obtained 5 or more days after the onset of fever can be used to distinguish between primary and secondary dengue infections. The presence of IgM antibodies to dengue virus in the absence of IgG antibodies indicates a primary infection, whereas when IgG antibody titres are higher than those of IgM, the presence of a secondary dengue infection is established. IgM antibodies acquired after infection with Japanese encephalitis virus (JEV) are relatively specific and can be used to differentiate between Japanese encephalitis and dengue infections. The ELISA, however, can be time consuming and not easily converted to a point-of-care-test (POCT). Recombinant flavivirus antigens are being introduced to identify IgM and IgG antibodies, but their specificity and sensitivity compared with that of inactivated whole virus have not been fully established.
RAPID DIAGNOSTIC TESTS

Lateral flow tests for dengue antibodies
Lateral flow tests for antibodies to dengue provide the same information as ELISAs. Tests using recombinant viral envelope glycoproteins of dengue viruses 1, 2, 3, and 4, respectively, have been introduced. These antigens are widely used in commercial kits. Careful studies are required to demonstrate sensitivity and specificity in diagnosing acute primary and secondary dengue infections and to differentiate between dengue, Japanese encephalitis, West Nile fever and yellow fever infections. When properly standardized, these tests offer an alternative to diagnostic tests using whole-virus antigens and should provide the clinician with a rapid and inexpensive method of distinguishing between acute and secondary dengue virus infections. Although lateral flow tests for dengue may suffer from lower sensitivity than ELISAs, they are true POCTs (rapid tests) and have several other advantages. Some of these features include ease of performance, speed, high stability with easy differentiation between primary and secondary infection using a single dilution of serum.

Lateral flow tests for dengue antigens
Lateral flow tests might be developed to capture whole virus, viral envelope antigens or nonstructural viral proteins in blood taken during the acute phase of infection. Such tests have been carried out experimentally, but have not been developed commercially. It is also possible that such tests could identify the specific infecting dengue virus, thus contributing important epidemiological data.

STRATEGIES FOR CONSIDERATION

It was determined by the participants in the workshop that the tests used for dengue should include those for primary care, ward care, hospital laboratory use, case surveillance and epidemiology. Furthermore, tests to determine disease severity, prognosis and vaccine evaluation were also considered to be important. The specifications of the ideal test for dengue, depending on purpose, are described below; the specifications were checked during and after the meeting with dengue experts. A strategy for prioritization of the various tests required for dengue was also established.

Specifications of the ideal test for dengue
Assuming that the ideal test for dengue has a high sensitivity and specificity, the first and second priorities for different uses include the following:

Clinical use
First priorities:
• early determination of acute dengue virus infection during the febrile phase;
• distinguishes between dengue and other flaviviruses;
• cheap;
• easy to use at all levels of the health system (rapid to perform).
Second priorities:
• remains positive after day 3 of symptoms (for patients who present at a late stage in the disease); however, the clinical manifestations of dengue can usually be recognized;
• if possible, provides an early marker of severe disease;
• distinguishes between first and subsequent infections;
• distinguishes between serotypes;
• stable at higher temperatures (greater than 30 °C), to be used at low-level healthcare units without refrigerators.

**Epidemiological surveillance**
First priorities:
• early determination of acute dengue virus infection during the febrile phase;
• remains positive after day 3 of symptoms (for patients who present at a late stage in the disease) and detects recent infection, although symptoms may have disappeared;
• distinguishes between dengue and other flaviviruses;
• distinguishes between serotypes (related to actual and also to past exposure, in order to determine rates of attack);
• cheap;
• easy to use at all levels of the health system (rapid to perform).
Second priorities:
• distinguishes between first and subsequent infections;
• stable at higher temperatures (above 30 °C);
• has a long shelf-life.

**Efficacy trials (vaccines and drugs)**
First priorities:
• distinguishes between dengue and other flaviviruses;
• distinguishes between serotypes;
• distinguishes between first and subsequent infections;
• gives a positive result throughout the whole period of illness.
Second priorities:
• acts as a marker of severe disease;
• easy to use.

**Type of test**
*Short-term tests.* Short-term tests include those for ward care, hospital laboratory and vaccine evaluation. It was suggested that for ward care the current IgM lateral flow test is adequate, although it was noted that the test generally gives negative results in the early clinical phase of the disease. For high throughput in hospital laboratories, ELISA was considered to be more suitable. For vaccine evaluations, it was suggested that the test for neutralizing antibody was most appropriate.

*Mid- to long-term tests.* For primary care, it was agreed that the detection of immune-complex dissociated NS1 antigen was best in a rapid test format. In addition to NS1 antigen, it was recognized that IgM and IgG antibodies to dengue should be detectable on the same lateral flow strip. Although type specificity was not required in this format, the need to distinguish dengue virus from the other flaviviruses was emphasized. Two additional detection systems for dengue were judged to be worth considering. One was a simple quantitative agglutination test, developed at Nagasaki University; the other test was under development at the Pasteur Institute. It was noted that neither the exact test configuration nor the performance characteristics of these systems were provided.

The implementation of molecular diagnostic tests, such as RT-PCR, in the hospital laboratory was considered to be important, having the potential to replace virus isolation. The present lack of standardization, complexity and higher costs would delay the widespread use of these tests. An attractive alternative to RT-PCR was real-time reverse transcription loop-mediated isothermal amplification assay
(RT-LAMP) for dengue. This method is faster, cheaper and more sensitive than the current molecular tests for dengue and could also be used for case surveillance, perhaps in conjunction with methods employing serotype-specific antigens and antibodies. It was considered that there was an important need for more definitive tracking of dengue using molecular epidemiological methods and that this should progress more quickly after standardization of molecular sequencing methods. Prospective studies on healthy volunteers would be required for these epidemiological studies.

Test evaluation

Short- and mid-term tests. It was suggested that there was a need for patient panels to be used in the detection of antibody, antigen and nucleic acid targets. These panels should be representative of well-defined primary and secondary infections, with all four serotypes of dengue included. In addition, patients should be categorized by age, day of illness, day of defervescence and severity of the disease. The proper use of these panels would require selection criteria and specifications for sample size.

It was suggested that the evaluation be performed in different geographic settings and that effectiveness should be assessed. It was emphasized that selection criteria and clear, standardized test protocols on the methodologies and the evaluation of test performance should be established prior to evaluation. It was expected that the World Health Organization (WHO) reference laboratories, along with other qualified reference laboratories, would be potential evaluation sites.

Long-term tests. It was suggested that a study should be carried out to evaluate the cost–benefit of the test systems for both the patient and health-care providers, as no data currently existed in this category. A comparison with the costs associated with vector control should be also provided. Furthermore, an assessment of the overall impact on public health should be performed.

ESTABLISHMENT OF REFERENCE LABORATORIES

Reagents

The identification of sources of standard reagents for immunodiagnostics and molecular diagnostics was considered to be an important requirement, and that blood banks could be involved in this programme. Although the acquisition of reagents from commercial suppliers was endorsed, it was noted that reagent sharing would be more restricted.

Quality assurance

Proficiency testing was stressed as an important goal; this testing could be organized by WHO as part of the Collaborating Centre recertification process via the WHO/Communicable Disease Surveillance and Response (CSR) Office in Lyon and could consist of a panel other than that used to establish new assays. It was suggested that each laboratory should use local specimens for this purpose. International standards for validation and quality control must also be used. Before establishing such a programme, there must be agreement on the reference “gold standard”. The importance of documentation was emphasized at this stage and it was expected that WHO would take responsibility for revising the manual on Diagnosis, treatment and prevention (WHO, 1997) at that time. Furthermore, assistance with good laboratory practice and good clinical practice should be provided by WHO.
Laboratory selection
It was noted that an early task would be to establish a list of laboratories willing to participate in a reference laboratory programme. The criteria for laboratory selection would be based on capacity, resources, past performance, scientific team, quality assurance/quality control capability and whether the facility was in an endemic area. The WHO Collaborating Centres involved should provide standardized protocols for testing. The Centres should also have easy access to reagents and a specimen repository.

Funding
Funding would be required for the basic activities of the reference laboratories; sources should include nongovernmental organizations, government and industry.

RESEARCH NEEDS
Research needs were considered to be mid- to longer-term goals.

New technologies on old platforms
It was recognized that new technologies could be evaluated in the context of existing technology platforms. This would shorten development time for the introduction of new and improved diagnostic tests for dengue. Some of the approaches that might be exploited are new antigen concentration methods and the use of non-infectious virus-like particles (VLPs). There was a need for the use of additional, recently described, recombinant dengue antigens to be thoroughly evaluated in old platforms.

Vaccine studies
A standard test for vaccine studies needs to be finalized. The tests under consideration were the neutralization and protection tests for determining efficacy.

Strain variability
Tests for strain variability must be established; these tests would have an impact on vaccine coverage, pathogenesis and diagnostic efficacy. To this end, a virus strain bank and a gene sequence bank should be established.

Prognostic markers
Given the limitations of the present immunological and nucleic acid-based tests, it was suggested that research into the development of new prognostic markers was required.

PROJECT ORGANIZATION AND FUNDING

Stakeholders and potential funding agencies
A key to the successful implementation of a comprehensive diagnostic programme for dengue was considered to be the organization and funding of projects. Early in the programme, it would be important to get the stakeholders (such as clinicians, public health authorities, medical device manufacturers and vaccine suppliers) involved, so that a workable plan could be established. The potential funding agencies, such as WHO, Pediatric Dengue Vaccine Initiative (PDVI), the Bill and Melinda Gates Foundation, National Institutes of Health (NIH) and other government
agencies would have to be contacted. It was agreed that there was a need for a “road map” to seek funding, and biannual meetings between the reference laboratories and WHO would be required in order to maintain good communication.

Leadership
The leadership required should be provided by WHO and should include stakeholders. It would be important for the Dengue Working Group and all other relevant divisions within the WHO to be involved in this programme.

Information network
It was suggested that an information network be established with funding derived from industry. The purpose of this network would be for the free exchange of information. It would also facilitate training. For example, the proper use of dengue POCTs could constitute an important element of this programme. The organization of this network would have to be defined by WHO. Finally, DengueNet needs to evolve to meet the needs of such a network.

Participation and training
Local government support would be required for local laboratory participation, and training should be supervised by the WHO Regional Offices.

NEXT STEPS
An overview of the main elements of the proposed UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR)/PDVI strategy for the development of improved tests for dengue is presented in Figure 1. More details were given in the presentation by R. Peeling (The role of WHO/TDR in facilitating the development, evaluation and application of diagnostics for dengue).

Reference

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1 http://www.who.int/GlobalAtlas/home.asp
**FIGURE 1.** Strategy for the development of improved tests for dengue: TDR/PDVI activities

- **Initiate organization & funding for development of new dengue tests**
  - Contact potential funding agencies
  - Provide leadership
  - Establish information network
  - Initiate participation & training

- **Define key research needs**
  - New technologies on old diagnostic platforms
  - Diagnostics for vaccine studies
  - Diagnostics for strain variability
  - Prognostic markers for severe dengue

- **Establish reference labs**

- **Identify companies for collaboration in test development**
  - Select test candidates for different purposes
  - Validate NS1 for early detection
  - Select labs, plan & initiate test evaluation
  - Establish guidelines for test usage
  - Decide on test format for clinical evaluation

- **Validate NS for early detection**
  - Validate existing POCTs

- **Select test candidates for different purposes**

- **Establish guidelines for test usage**

- **Select labs, plan & initiate test evaluation**

- **Decide on test format for clinical evaluation**

- **Letters of interest, selection & approval**
  - Secure funding
  - Obtain reagents
  - Establish QA

**NS1, nonstructural glycoprotein 1; PDVI, Pediatric Dengue Vaccine Initiative; POCT, point-of-care test; QA, quality assurance**
Goals of the workshop

Scott B. Halstead
Director of Research, Pediatric Dengue Vaccine Initiative, Bethesda, Maryland, USA

Dengue is a huge global infectious disease problem. Dengue viruses, in many instances all four serotypes, are transmitted in about 110 tropical and subtropical countries, with the combined at-risk population approaching 3.0 billion. Studies of dengue seroprevalence around the world suggest that average annual rates of infection with dengue in much of this population could be between 5 and 10%. A conservative estimate, which does not accurately reflect second or third infections in the same individual, is that 50–100 million dengue infections occur annually. A small, but irregular, fraction of all dengue infections are symptomatic. The ratio of symptomatic to asymptomatic infections approaches 1 for primary infections with dengue virus-1 (DENV-1), or secondary infections with DENV-2 or -3. Other infections or infection sequences produce mild disease or are largely inapparent.

Mild dengue syndromes are difficult to distinguish from a long list of other viral, bacterial or parasitic infections. For optimal management, the dengue shock syndrome requires a high index of suspicion on the part of parent and physician, a learning experience that requires rapid, high-quality serological identification. The laboratory diagnosis of dengue is increasingly important for many reasons, including the size of the dengue problem, the complexity of differential diagnosis for the physician, the need to make an early diagnosis in order to institute life-saving resuscitation, plus utility in public health surveillance and monitoring disease control. These factors have created a huge market that is beginning to be met by commercial diagnostic tests and reagents. The fact that dengue diagnostics are marketed across national borders means that there is no agency designated to control test quality. Three decades ago, WHO organized an informal quality-control system, which functioned mostly by exchange of reagents and test methods. At that time, the Yale Arbovirus Research Laboratory served as a quality-control laboratory providing virus identification services, standard antigens and antisera in limited quantities, and technical training. Subsequently, the Center for Disease Control Laboratory for Vector Borne Infectious Diseases at Fort Collins assumed this role. The demand for standardization and quality control in dengue diagnostics has far outstripped these earlier programmes. WHO is the only agency with the mandate to meet the international quality control requirements for these important diagnostics. Against this background, the goals of this workshop were:

To review existing dengue diagnostics with respect to:
• Standardized tests and reagents
• Subsidized test reagents
• Dengue strain repository
• Quality control of commercial tests
• Improved tests to detect antigens and antibodies in terms of:
  – Sensitivity
  – Specificity
  – Cost
Given the circulation of four dengue viruses in some geographic areas, with the concurrent circulation of other flaviviruses e.g. Japanese encephalitis virus or vaccine, West Nile or yellow fever virus or vaccine, the diagnosis of dengue infection must be accurately made in the following scenarios:

- acute illness; primary infection; detect antigen, virus or RNA specifically, rapidly and cheaply;
- acute illness; secondary infection; detect antigen, virus, RNA of current infection specifically, rapidly and cheaply; detect antibody for first infection specifically;
- early convalescence; primary infection; detect antigen, RNA or antibody specifically, rapidly and cheaply;
- early convalescence; secondary infection; detect current infecting antigen, RNA or antibodies to current infection; detect antibodies to second, third or fourth infection specifically;
- late presentation after overt or inapparent infections, identify all lifetime flavivirus infections specifically; this is the ‘holy grail’ of dengue serology.

To illustrate the problems associated with currently available tests, the interpretative difficulties posed by five actual sets of data were discussed (Table 1).

Each of these cases is from the Kam Phaengphet cohort study for which neutralization tests are performed at the Armed Forces Research Institute of Medical Sciences (AFRIMS). Sera were obtained from children before and after the rainy season.

Case No. 298: DENV-1 was isolated during the acute phase illness in a child immune to dengue-2; the child, as a result, either developed an anamnestic JE neutralizing antibody response or experienced infections with dengue-1 and JE during the observation period. No test is available to select between these options.

Case No. 636 is a child whose prior dengue virus infection experience resulted in a high titre of neutralizing antibodies to several dengue viruses, including DENV-3. Despite these antibodies, this child experienced a infection with dengue-3. In a subsequent test using the homologous DENV-3 virus in the same laboratory, pre-infection antibodies to DENV-3 disappeared.

Case No. 132 is a child with immunity to multiple dengue viruses who apparently experienced a JE infection.

Case No. 149 is a child with a low titre of antibodies to DENV-2 who was infected either by a dengue virus or by dengue plus JE; however, as no virus was isolated, the etiology of infection cannot be surmised.

Finally, case No. 158 is a child with antibodies to JE who experienced a specific boost in neutralizing antibodies. Is this a wild-type JE infection in a child previously given JE vaccine? Although this is quite likely, the serological tests necessary to verify this hypothesis are not available.
### TABLE 1. Examples of difficulties in interpretation of results obtained from dengue diagnostics (five cases)

<table>
<thead>
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<th>Case No.</th>
<th>Date</th>
<th>Test</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>JE</th>
<th>Virus isolation</th>
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<td>1013</td>
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</tbody>
</table>

D1, D2, D3, D4, Dengue serotypes 1 to 4; JE, Japanese encephalitis
Laboratory diagnosis of dengue
IMMUNOLOGICAL RESPONSES TO DENGUE INFECTIONS

David Vaughn
Military Infectious Diseases Research Program, United States Army Medical Research and Materiel Command, Fort Detrick, Maryland, USA

Immunological responses to dengue virus infection include innate and acquired responses. These responses influence virus replication/clearance and disease severity (subclinical infections through life-threatening plasma leakage and haemorrhage). Dengue viraemia and antibody response patterns are most readily used for the laboratory diagnosis of dengue virus infection and are the focus of this discussion. The degree of viraemia may have implications for case management as the peak of dengue viraemia early in the febrile phase has been shown to correlate with ultimate disease severity.

Dengue viraemia appears to be universal in febrile patients with dengue; it occurs prior to the onset of fever and symptoms and peaks 2–3 days after the onset of illness, which is typically 2–3 days before defervescence (Figure 2). A diagnosis of acute infection with dengue virus can be made by isolating the virus from cell culture, live mosquitoes or animals, or by detecting viral genome or antigen. The time taken to carry out tests using these detection methods can be hours to weeks. Several approaches to RT-PCR have been published, with commercial kits soon to be available. On average, the pattern of viraemia follows the fever curve (Figures 3A and 3B). Clearance of culturable virus from the blood coincides with defervescence. A diagnosis of dengue based on virus detection is difficult after defervescence.

Serologically, a primary infection with dengue virus results in detectable levels of IgM antibodies by the third afebrile day after infection (Figures 3A and 3B). These IgM antibodies persist for 1–2 months after infection. Secondary infections with dengue virus are characterized by a rapid increase in IgG antibody levels, with more

FIGURE 2. Mean viraemia titre by day of defervescence in 47 Thai children having a secondary infection with DENV-2
FIGURE 3A. Virus and antibody response patterns in Thai children with primary dengue virus infections

Primary dengue, virus/antibody patterns (n=66)

FIGURE 3B. Virus and antibody response patterns in Thai children with secondary dengue virus infections

Secondary dengue, virus/antibody patterns (n=246)
modest increases in IgM (Figures 3A and 3B). Owing to the relatively late increase in antibody levels to a concentration that can be detected diagnostically, a negative result for an antibody test early in the course of disease is not definitive. Specimens should be collected at least 7 days after the onset of symptoms in order to rule out the possibility of an acute infection with dengue virus. While IgM antibodies produced during a dengue virus infection have the highest activity against the infecting serotype, the IgM antibodies are also cross-reactive with other dengue virus serotypes and to a lesser degree, other flaviviruses. Ideally, IgM antibody that recognizes dengue virus or virus antigen should be quantitatively compared with other flaviruses that circulate in the geographical area.

Antibodies produced during dengue virus infection provide short-lived protection against infection with a heterologous serotype of dengue virus. Neutralizing antibody levels correlate with protection for many viral infections; this may also be the case for dengue. The presence of measurable levels of dengue antibody is generally protective, with the exception of low levels of cross-reactive antibodies induced by a virus of different serotype than the infecting type. In this situation, the antibody can conceivably enhance virus replication and the severity of disease manifestations (according to the immune enhancement theory of dengue pathogenesis).

In summary, current approaches to the laboratory diagnosis of acute infection with dengue virus are dependent upon the detection of virus before defervescence and the detection of antibody after defervescence. The ideal assay for the diagnosis of acute disease would be used at the bedside or clinic laboratory and would be able to detect both virus and the acute-phase antibody responses that distinguish between primary and secondary dengue virus infections.

Reading list
TESTING FOR DENGUE: AN OVERVIEW

Vance Vorndam
Centers for Disease Control and Prevention, Dengue Branch, San Juan, Puerto Rico

The diagnosis of flavivirus infections has come a long way since the days when haemagglutination inhibition (HAI), complement fixation and mouse inoculation were among the few options available. Today, new techniques for measuring selected parts of the immune response and for detecting viruses and subviral components are being combined with automated techniques to make diagnosis more specific, sensitive and rapid. For the purposes of this workshop, it is suggested that two goals for the diagnostic laboratory are paramount. First, clinicians need to be able to obtain a rapid and specific diagnosis from acute-phase samples in order to facilitate patient management. Second, for vaccine development, a definitive characterization of immune status is essential. However, some characteristics of dengue virus infections make these goals difficult. From a virology stand-point, viraemia levels are usually falling by the time the patient visits a doctor, making virus isolation difficult at an early stage, and impossible after a week of illness. Second, IgM antibody appears late in the acute phase and does not reach a peak until a week after onset. Finally, in secondary flavivirus infections, common in adults, the broad cross-reactivity of the immune response nullifies the specificity of most serological tests.

The standard test for virus isolation and identification involves cell culture, usually of the C6/36 mosquito cell line, combined with fluorescence-labelled antibodies of various specificities. Where insectary facilities are available, mosquito inoculation improves the sensitivity of virus detection. These techniques are now being supplanted by a variety of techniques for the amplification of nucleic acid. Conventional polymerase chain reaction (PCR), in which the products of amplification are usually detected by gel electrophoresis, is now being supplanted by ‘real-time’ PCR, wherein products are detected by more sensitive fluorescent markers. Other variations on amplification techniques, such as NASBA, are becoming increasingly popular owing to their relative simplicity and the availability of standardized kits.

ELISAs for IgM and IgG antibodies are the standards for the serological analysis of dengue virus infections, as they are simple and allow large numbers of samples to be tested. A limitation of these tests is that specificity is generally lacking, particularly in the case of secondary infections. This problem can be partially, but not completely, addressed by virus neutralization tests, such as the plaque-reduction neutralization test and newer ELISA-format tests.

Future research needs to be directed towards three areas. First, increased sensitivity will be attained by developing improved techniques for amplification and new fluorescence systems. Second, increased specificity will be found by developing specific antigens and modified ELISAs. Finally, the use of automation will facilitate the testing of large numbers of samples in a time frame that will be useful at the time in which the information is most needed.
LABORATORY DIAGNOSIS OF DENGUE INFECTION: EPIDEMIOLOGY AND FIELD STUDIES

Maria G. Guzmán, Mayling Alvarez, Susana Vazquez and Gustavo Kouri
Virology department, PAHO/WHO Collaborating Center for Viral Diseases, 'Pedro Kouri' Tropical Medicine Institute, Havana, Cuba

Introduction
The accurate and efficient diagnosis of dengue is important for clinical care and surveillance support. Serology is the most widely applied method used in routine diagnosis (Guzmán & Kouri, 2004). It is possible to identify a recent or past infection with dengue on the basis of the relative persistence of anti-dengue antibodies. In this paper we discuss the usefulness of serological assays in the study of recent and past infections.

Serological assays for dengue

Detection of recent transmission of dengue
Seroepidemiological studies can be used to define recent infections, and to confirm the transmission of dengue or an increase in its incidence in areas where dengue is not endemic (where Aedes aegypti is present) or in endemic countries during inter-epidemic periods (PAHO, 1994).

Different serological markers can be used, the most widely employed being anti-dengue IgM, which is used as a marker of recent infection. The presence of anti-dengue IgG in the serum is a criterion for past infection with dengue; however, the presence of a high titre of IgG in a single sample of serum suggests a recent infection, while seroconversion or a fourfold increase in the titre of IgG in paired sera from a patient with suspected dengue confirms infection (Guzmán & Kouri; 2004; Vorndam & Kuno, 1997).

Capture ELISA to detect anti-dengue IgM, and HAI assay or ELISA to detect a high level of anti-dengue IgG are useful methods for detecting dengue infection in samples of serum or blood collected on filter papers from febrile cases, patients with fever and rash, and cases of suspected dengue (patients with fever and two or more symptoms of dengue fever, such as myalgia, arthralgia, headache, and rash). The results of tests performed in the laboratory must be complemented with the appropriate clinical and epidemiological information (PAHO, 1994; Gubler, 1996; Vorndam & Kuno, 1997; Guzmán & Kouri, 2004).

The usefulness of these studies will depend upon the epidemiological situation, the capacity of the surveillance system to detect the first cases of the outbreak, and the rapidity with which effective vector control measures can be established.

Good examples illustrating the usefulness of sero-epidemiological studies in the early detection of dengue transmission in non-endemic areas where low indexes of Ae. aegypti are present include the outbreaks of dengue in Santiago de Cuba in 1997 and in Havana in 2000. Studies on these outbreaks were crucial for the rapid implementation of effective vector control measures (Kouri et al., 1998; Guzmán & Kouri, 2002; Pelaez et al., 2004).

At the end of 1996, several risk factors for dengue transmission were reported in the municipality of Santiago de Cuba; an active surveillance system was established in mid January 1997, following the guidelines for the prevention and control of dengue and dengue hemorrhagic fever (PAHO, 1994). An active search was made, via the primary health-care system, for febrile patients and patients suspected to...
have dengue. Samples of sera collected 5 to 6 days after the onset of fever were assayed for anti-dengue IgM. On January 28, seven cases of dengue were detected in one health area of the municipality. Once clinical cases were detected, within 2 days IgM capture ELISA and IgG ELISA (ELISA inhibition method) confirmed dengue infection in 12 cases, seven of them with a secondary infection (Figure 4) (Vazquez et al., 1997; Vazquez et al., 1998). A retrospective sero-epidemiological study in 592 clinically compatible cases of dengue indicated that none had anti-dengue IgM antibodies, suggesting that initial transmission had occurred during the second half of December 1996, and it was therefore highly probable that the cases detected in January were the first (Valdes et al., 1999).

In contrast, the index case in the outbreak of dengue in Havana in 2000 was confirmed by detection of anti-dengue IgM and a high titre of anti-dengue IgG on September 15. The index case had an onset date of September 1. In a sero-epidemiological retrospective study to look for any patients with dengue fever or undifferentiated fever including cohabitants, conducted within a perimeter of 300 m around the domicile of the index case, six individuals were confirmed as being infected with dengue, five of them having an asymptomatic infection. Once dengue transmission was detected, a prospective surveillance study was established to look for cases of suspected dengue around the index case. Using the same serological assays, dengue infection was confirmed in eleven febrile cases with a date of onset of fever of September 6 to 21. Epidemiological studies around these patients suggested that the first cases occurred in late August (Guzmán & Kouri, 2002; Pelaez et al., 2004).

In both epidemic situations, active surveillance allowed health authorities to be alerted at an early stage. Once dengue had been detected, serological surveillance allowed the epidemics to be characterized and the cessation of transmission to be confirmed.

During the Havana outbreak, 138 cases of dengue were confirmed. The epidemic was eliminated in 3 months. In contrast, despite early detection, the 1997 outbreak could not be eliminated in the first weeks; however, transmission was restricted to this municipality, with no autochthonous cases in the rest of the provinces. In total, 3012 cases of dengue were confirmed.

**FIGURE 4.** First cases of dengue detected during the outbreak of dengue-2 in Santiago de Cuba, 1997

[Diagram showing the outbreak of dengue-2 in Santiago de Cuba, 1997 with dates of onset of fever for different cases.]

*Dates refer to date of onset of fever*
Detection of a past infection with dengue
The study of a past infection with dengue allows the determination of:
• the incidence of the infection after epidemics;
• the prevalence of antibodies to the viruses;
• the level of immunity after vaccination; and
• risk factors for severe disease.

Population-based community serosurveys conducted shortly after epidemics (incidence studies) or years after transmission, and retrospective and prospective studies in cases of dengue fever (and dengue haemorrhagic fever) can be performed. In these investigations, the measurement of anti-dengue IgG in samples of both sera and blood collected on filter papers provides useful information.

Longevity of antibodies
The duration of immunity to infection with dengue is not well known. Retrospective studies have determined the presence of neutralizing anti-dengue antibodies in samples of serum from persons infected 40 or more years previously (Papaevangelou & Halstead, 1977; Mas et al., 1979; Tadano et al., 1983; Guzmán et al., 2000). Table 2 shows some examples of dengue neutralizing antibodies in individuals infected during massive epidemics. Retrospective studies done by Halstead in 1974 and Okuno et al. in 1983 in individuals inoculated 40 or more years previously support these observations (Halstead, 1974; Okuno et al., 1983). The persistence of neutralizing antibodies for more than four decades in studied persons suggests that homotypic immunity to dengue virus can last for life, but the kinetics and longevity of both homotypic and heterotypic neutralizing antibodies are not well characterized.

Assays for detecting a past infection
Assays based on HAI, IgG ELISA and neutralization are useful for detecting a past infection with dengue.

HAI, while sensitive and reproducible, requires that sera are treated with acetone or kaolin to remove inhibitors, and with goose red blood cells to eliminate non-specific agglutinins. This assay is satisfactory for identifying infection with a flavivirus, however, it lacks the specificity necessary to distinguish the type of flavivirus involved (Clarke & Casals, 1958; Vorndam & Kuno, 1997; Guzmán & Kouri, 2004).

However, the presence of serum antibodies by HAI does not prove prior infection with the test antigen virus, but only provides evidence for past infection with an unspecified flavivirus (Burke, Nisalak & Gentry, 1987). Although the HAI assay

<table>
<thead>
<tr>
<th>Virus</th>
<th>Location</th>
<th>Year</th>
<th>Interval (years)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV-1 &amp; DENV-2</td>
<td>Athens</td>
<td>1928</td>
<td>&gt; 45</td>
<td>Papaevangelou et al. (1977)</td>
</tr>
<tr>
<td>DENV-1</td>
<td>Osaka</td>
<td>1944/45</td>
<td>&gt; 36</td>
<td>Tadano et al. (1983)</td>
</tr>
<tr>
<td>DENV-1 &amp; DENV-2</td>
<td>Havana</td>
<td>1945</td>
<td>&gt; 30</td>
<td>Mas et al. (1979)</td>
</tr>
<tr>
<td>DENV-1</td>
<td>Santiago de Cuba</td>
<td>1998</td>
<td>20</td>
<td>Guzmán et al. (2000)</td>
</tr>
</tbody>
</table>
is still the gold standard technique allowing the serological confirmation of a flavivirus infection (if seroconversion of antibodies is observed in paired samples of sera), it is not currently employed as a routine diagnostic tool.

IgG ELISA has replaced the HAI assay because it has the potential to be automated and thus can accommodate a large number of samples. In addition, no processing of the serum is required and only a few microlitres of the sample are needed (Vorndam & Kuno, 1997; Guzmán & Kouri, 2004). Antigens prepared in mouse brain or in tissue culture can be used. Several formats of immunoenzymatic assays for the detection of anti-arbovirus antibody have been described, including indirect, capture IgG, the inhibition method, and double antibody sandwich ELISA (Oseni et al., 1983; Tadeu, Figueiredo & Shope, 1987; Vazquez et al., 1997; Miegostovich et al., 1999). As with HAI, IgG antibodies detected by ELISA are usually cross-reactive, even during a primary infection with dengue.

The neutralization assay is considered to be the serological technique with the greatest specificity, allowing differentiation between flaviviruses and determination of neutralizing antibodies to the four dengue viruses (Russell & Nisalak, 1967). Some important aspects related to the neutralization assay are:

- Neutralizing antibodies are directed to the envelope and membrane virus proteins.
- The specificity of neutralizing antibodies increases over time. Samples of serum collected months or years after infection are preferred for study.
- A low level of cross-reactivity is observed in a primary infection with dengue.
- The detection of neutralizing antibodies to several serotypes suggests that multiple infections with dengue have occurred.
- In general, a low level of cross-reaction to different flaviviruses is observed.

Some authors have adopted a stringent criterion in order to define the infecting serotype. Sangkawibha et al. (1984) considered a test result to be positive for dengue virus when a reduction of 70% or greater in the formation of plaques was observed at a 1 in 30 dilution of serum. The bias inherent in this criterion is not known, considering that most of the serological studies have been done in areas where dengue viruses co-circulate. The epidemiological situation in Cuban is probably unique in terms of provision of consistent data. After a widespread epidemic of dengue fever caused by DENV-1 in 1977, a second epidemic caused by DENV-2 occurred in 1981 (Kouri et al., 1989). Both outbreaks involved the whole country. After 15 years without dengue transmission, an outbreak of dengue-2 was observed in 1997 in one municipality of the country; this outbreak was eliminated in 7 months and did not extend to the rest of the provinces (Kouri et al., 1998). Finally, after a small outbreak, an epidemic caused by DENV-3 occurred in in 2001–2002 and affected mainly Havana (the capital city) (Guzmán & Kouri, 2002; Pelaez et al., 2004). All these outbreaks were eliminated and no endemicity has been reported.

Different retrospective and prospective serological studies in the open population and in clinical cases have been conducted to study the Cuban epidemics, to determine the role of secondary infection as a risk factor for dengue haemorrhagic fever and to determine antibody prevalence and incidence of infection after epidemics. A reduction of 50% or more in plaque formation by dengue virus at a serum dilution of 1 in 30 was the criterion employed in these studies.

In sera collected before and after the 1981 epidemic caused by DENV-2, it was demonstrated that use of a 1 in 30 dilution of serum allowed discrimination between neutralizing antibodies to DENV-1 and DENV-2 (Table 3) (Guzmán et al., 1991). After a primary infection, a monotypic reaction is generally observed to the infecting virus. In contrast, after a secondary infection the IgG response is broadly
cross-reactive and may result in higher neutralization titres to the first of the sequential virus serotypes (the ‘original antigenic sin’ phenomenon) (Halstead, Rojanasuphot & Sangkawibha, 1983; Vorndam & Kuno, 1997); in individuals infected sequentially with dengue virus, levels of antibodies are greatest to the original infecting virus serotype.

The plaque reduction neutralization technique (PRNT) is the most specific and traditional serological tool. Several protocols using agar, agarose or carboxymethylcellulose, and different cell cultures, such as African green monkey kidney cells (Vero), hamster kidney cells (BHK21), rhesus monkey kidney cells (LLCMK₂), and porcine kidney cells (PS), have been employed (Morens, Halstead & Larsen, 1985; Morens DM et al., 1985; Guzmán & Kouri, 1996; Jirakanjanakit et al., 1997). In this test, diluted serum is incubated with defined amounts of the viruses giving the neutralizing antibody titre. The end-point of the titration is the highest dilution of serum that reduces the number of plaques by 50 to 90%. On some occasions, a single dilution of serum is employed to detect the presence of neutralizing antibodies to a particular virus.

In addition to PRNT, some researchers have employed a microfocus reduction neutralization test (based on the peroxidase-antiperoxidase technique) and a micro-neutralization test based on an ELISA format (Jirakanjanakit et al., 1997; Vorndam & Beltran, 2002).

PRNT is labour-intensive, time-consuming and expensive. It also requires good standardization in order to obtain results that are reproducible and comparable between laboratories.

Three important aspects deserve careful attention: the selection of the viral strain to be employed, its passage number, and the biological system of passage can influence the results obtained. Recently, Kochel et al. (2002) tested sera from Peruvian individuals previously infected by DENV-2 of the American genotype. It was remarkable that the level of specific antibodies to the infecting strain were higher.

### Table 3. Reciprocal of neutralizing antibody titre to DENV-1 and DENV-2 in sera collected before and after the Cuban epidemic of dengue-2 in 1981

<table>
<thead>
<tr>
<th>Year of sample collection</th>
<th>DENV-1</th>
<th>DENV-2</th>
<th>Type of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980</td>
<td>160</td>
<td>&lt;10</td>
<td>Primary dengue-1</td>
</tr>
<tr>
<td>1980</td>
<td>250</td>
<td>&lt;10</td>
<td>Primary dengue-1</td>
</tr>
<tr>
<td>1980</td>
<td>76</td>
<td>24</td>
<td>Primary dengue-1</td>
</tr>
<tr>
<td>1980</td>
<td>320</td>
<td>26</td>
<td>Primary dengue-1</td>
</tr>
<tr>
<td>1980</td>
<td>42</td>
<td>22</td>
<td>Primary dengue-1</td>
</tr>
<tr>
<td>1983</td>
<td>90</td>
<td>40</td>
<td>Secondary dengue-1 &amp; 2</td>
</tr>
<tr>
<td>1983</td>
<td>60</td>
<td>48</td>
<td>Secondary dengue-1 &amp; 2</td>
</tr>
<tr>
<td>1983</td>
<td>&lt;10</td>
<td>82</td>
<td>Primary dengue-2</td>
</tr>
<tr>
<td>1983</td>
<td>&lt;10</td>
<td>70</td>
<td>Primary dengue-2</td>
</tr>
<tr>
<td>1983</td>
<td>&lt;10</td>
<td>130</td>
<td>Primary dengue-2</td>
</tr>
</tbody>
</table>

Reproduced and adapted from: Guzmán MG et al. 1991, with permission from Memorias dos Institutos Oswaldo Cruz

* Reciprocal of neutralizing antibody titre as determined by plaque reduction neutralization assay
than those observed for a different strain of the same serotype belonging to the Asian genotype. Table 4 shows the geometrical mean of antibodies determined in this study.

Probably much more important is the fact that the direct correlation between neutralizing antibodies determined by these neutralization assays in vitro with the neutralizing capacity of these antibodies in vivo is not known.

PRNT is a useful tool when used correctly. It has been performed in several sero-epidemiological surveys in Cuban donors to obtain important information such as the prevalence of antibody to the infecting virus and to support the role of the secondary infection in severe disease (Guzmán et al., 1990; Guzmán et al., 2000). Moreover, in these studies it has been possible to measure the silent transmission of dengue during an epidemic of dengue haemorrhagic fever, the role of age in the course of a secondary infection and the role of the virus sequence interval, all of which are considered today as risk factors for dengue haemorrhagic fever and dengue shock syndrome (Guzmán et al., 2000; Guzmán et al., 2002a, 2002b).

Conclusions
The detection of antibodies to dengue is useful for detecting a recent or a past infection. Different tools, such as HAI assay, ELISA and PRNT have been employed, the latter being the most specific. However, an easy, reproducible, inexpensive and rapid test for the detection of neutralizing antibodies, which would give specific results without diminishing sensitivity, is still required. Studies must be designed to define the correlation of in vitro and in vivo results.

References

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**TABLE 4.** Geometric mean titre of neutralizing antibodies to DENV-2 strains in sera from Peruvian individuals

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Strain</th>
<th>Geometric mean titre</th>
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<tbody>
<tr>
<td>American</td>
<td>IQT2124</td>
<td>4523</td>
</tr>
<tr>
<td></td>
<td>IQT2913</td>
<td>417</td>
</tr>
<tr>
<td>Asian</td>
<td>16681</td>
<td>635</td>
</tr>
<tr>
<td></td>
<td>OBS8041</td>
<td>335</td>
</tr>
</tbody>
</table>

From Kochel et al. (2002)


Laboratory diagnosis of dengue using commercial kits
COMMERCIAL KITS AND REAGENTS

Roy R. Mondesire
TDR, Diagnostics Research and Development, WHO, Geneva, Switzerland

A list of the commercially available, easy-to-use tests for the diagnosis of patients with suspected dengue infection is presented in Table 5. This work is in progress; please note that although all of the suppliers listed were contacted, some did not provide complete product information. The first column of the table contains details of the companies involved. Several companies listed in earlier drafts have been removed because of evidence for the discontinuation of the products, or non-existent web sites, e-mail addresses or telephone numbers. Further updates will be required as changes occur and as more information becomes available.

<table>
<thead>
<tr>
<th>Company</th>
<th>Tests for dengue</th>
<th># of tests per kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ameritek USA</td>
<td>R84-110, Dengue IgG; serum (strip)</td>
<td>LF 50</td>
</tr>
<tr>
<td></td>
<td>R84-112, Dengue IgG; serum (cassette)</td>
<td>LF 30</td>
</tr>
<tr>
<td>Mill Creek, WA 98012, USA</td>
<td>R85-110, Dengue IgM; serum (strip)</td>
<td>LF 50</td>
</tr>
<tr>
<td>(<a href="http://www.ameritek.org/product.htm">http://www.ameritek.org/product.htm</a>)</td>
<td>R85-112, Dengue IgM; serum (cassette)</td>
<td>LF 30</td>
</tr>
<tr>
<td>BIO-RAD Laboratories</td>
<td>NS1 Detection System: under evaluation</td>
<td>NA NA</td>
</tr>
<tr>
<td>R&amp;D Manager, Clinical Microbiology Division</td>
<td>3, Boulevard Raymond Poincaré, 92430 Marnes-la-Coquette, France</td>
<td></td>
</tr>
<tr>
<td>(<a href="http://www.bio-rad.com/">http://www.bio-rad.com/</a>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinotech Diagnostics &amp; Pharmaceuticals</td>
<td>Dengue Fever cassette test single pouched</td>
<td>LF NA</td>
</tr>
<tr>
<td>2101–11871 Horseshoe Way</td>
<td>Dengue Virus IgG</td>
<td>ELISA 96</td>
</tr>
<tr>
<td>Riverside Industrial Park</td>
<td>Dengue Virus IgM</td>
<td>ELISA 96</td>
</tr>
<tr>
<td>Richmond, BC V7A 5H5, Canada</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(<a href="http://www.clinotech.com/tech.htm">http://www.clinotech.com/tech.htm</a>)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortez Diagnostics</td>
<td>173106-1, OneStep Dengue Fever IgG &amp; IgM RapiCard™ InstaTest (cassette, WB/serum)</td>
<td>LF 20</td>
</tr>
<tr>
<td>23961 Craftsman Road, Suite E/F</td>
<td>173105-1, OneStep Dengue Fever IgG RapiCard™ InstaTest (cassette, serum test); bulk or boxed</td>
<td>LF 25/50</td>
</tr>
<tr>
<td>Calabasas, CA 91302-2521, USA</td>
<td>173107-1, OneStep Dengue Fever IgG RapiDip™ InstaTest (strip, serum test); bulk or boxed</td>
<td>LF 25/50</td>
</tr>
<tr>
<td>(<a href="http://www.rapidtest.com">http://www.rapidtest.com</a>)</td>
<td>175105-1, OneStep Dengue Fever IgM RapiCard™ InstaTest (cassette, serum test); bulk or boxed</td>
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<td>175108-1, OneStep Dengue Fever IgM RapiDip™ InstaTest (strip, serum test); bulk or boxed</td>
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<tr>
<td></td>
<td>173104-1, OneStep Dengue Fever IgG/IgM RapiCard™ InstaTest (cassette, WB/serum test); bulk or boxed</td>
<td>LF 25/50</td>
</tr>
<tr>
<td>Cypress Diagnostics</td>
<td>344 Dengue Quick Test IgG/IgM</td>
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<tr>
<td>Langdorpsesteenweg 160</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-3201 Langdorp, Belgium</td>
<td></td>
<td></td>
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<tr>
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<td>Diazyme Laboratories</td>
<td>DZC003a Dengue Virus IgG</td>
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<td>Diazyme Laboratories</td>
<td>DZC003b Dengue Virus IgM</td>
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<tr>
<td>PO Box 85608</td>
<td>DZC0012</td>
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<tr>
<td>San Diego, CA 92186-5608, USA</td>
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<td>(<a href="http://www.diazyme.com/">http://www.diazyme.com/</a>)</td>
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(Table continues on next page)
(Continued from previous page)

<table>
<thead>
<tr>
<th>Company</th>
<th>Tests for dengue</th>
<th>Type</th>
<th># of tests per kit</th>
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<tbody>
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<td><strong>Focus Diagnostics, Inc.</strong></td>
<td>EL1500G Dengue Fever Virus ELISA IgG</td>
<td>ELISA</td>
<td>96</td>
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<tr>
<td></td>
<td>EL1500M Dengue Fever Virus IgM Capture ELISA</td>
<td>ELISA</td>
<td>96</td>
</tr>
<tr>
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<td>31211-050 Dengue BLOT IgG</td>
<td>Blot</td>
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<td>31221-020 Dengue IgM BLOT IgM</td>
<td>Blot</td>
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<tr>
<td></td>
<td>A dot-blot assay incorporating major immunogenic antigens from all four serotypes for the detection of IgG antibodies to dengue virus.</td>
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<tr>
<td><strong>Hema Diagnostics</strong></td>
<td>Dengue diagnostics/NS5 detection: under evaluation</td>
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<td>LF</td>
<td>25</td>
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<td></td>
<td>184M Dengue IgM, WB/serum</td>
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<tr>
<td></td>
<td>Dengue IgM Catalogue No. 181M</td>
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<td>96</td>
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<td>Dengue Fever IgG &amp; IgM Combo</td>
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<tr>
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<td>Dipstick (Catalogue No. ML010-014A)</td>
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<td></td>
<td>Device (Catalogue No. ML010-014C)</td>
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<td>OD227, Dengue M</td>
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<td><strong>Orgenics</strong></td>
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<td>Comb</td>
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<td><strong>PanBio</strong></td>
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<td>DCG-100 (EDEN02G) – Dengue IgG Capture</td>
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<td>DEM-200 (E-DEN01M) – Dengue IgM Capture</td>
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<td>EC-400 (E-DEN-01D) DUO Dengue IgM &amp; IgG Capture</td>
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<td>R-DEN02D IgG/IgM</td>
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<td><strong>Standard Diagnostics Inc.</strong></td>
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<td></td>
<td>E-102-A Dengue IgG &amp; IgM card test</td>
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<td>25</td>
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<td><strong>Tulip Group</strong></td>
<td>Denguecheck 50200010</td>
<td>LF</td>
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<tr>
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<td>Denguecheck 50200025</td>
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<td>25</td>
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ELISA, Enzyme-linked immunosorbent assay; LF, Lateral flow (test is based on immunochromatography); NA, Not available; WB, Whole blood
DIAGNOSTIC ACCURACY OF RAPID IMMUNOCROMATOGRAPHIC ASSAYS FOR THE DETECTION OF IGM ANTIBODIES TO DENGUE VIRUS DURING THE ACUTE PHASE OF INFECTION:
A SYSTEMATIC REVIEW AND META-ANALYSIS

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Introduction
Laboratory testing plays an important role in the diagnosis of dengue via the detection of dengue-specific antibodies or antigens (Gubler, 1998; Guzmán & Kouri, 2004). Serological tests for the identification of dengue infection rely on the detection of IgM antibodies during the acute phase of infection, either (1) a fourfold rise in antibody titre in paired serum collections, or a single serum with an antibody titre of ≥ 1:2560 measured by HAI assay; or (2) a positive result in an IgM antibody capture ELISA (MAC-ELISA) (WHO, 1997; Guzmán & Kouri, 2004). In recent years, the development of rapid assays has allowed patient specimens to be tested in point-of-care situations, such as a doctor’s office or hospital outpatients’ department (Price, 2001). Such assays are often referred to as immunochromatographic tests (ICTs); simple and quick to perform, these assays detect anti-dengue IgM/IgG antibodies in serum or plasma samples (Vaughn et al., 1998). Many manufacturers of ICTs for dengue also claim that their tests are able to detect and differentiate between primary and secondary infections with dengue. However, despite numerous studies comparing dengue ICTs with reference assays, the diagnostic accuracy of such assays has not been reliably established because of the multiplicity of methodologies used in evaluation (Shu & Huang, 2004). Before dengue ICTs can be used confidently for the diagnosis of infection with dengue, a systematic review and meta-analysis of study results is required in order to establish the diagnostic accuracy of these tests.

We have carried out such a review and meta-analysis, with the following aims: (1) to review the available literature for rapid (less than 60 min) point-of-care assays for the diagnosis of acute infection; and (2) to perform a meta-analysis of the results of rapid point-of-care dengue assays for the diagnosis of acute dengue infection when compared with those of reference assays.

Materials and methods

Literature search and study retrieval. Several approaches were used to ensure that relevant studies were identified. The primary means of identifying suitable studies was use of Internet-based search engines. Searches were conducted on open-access databases (MEDLINE² 1966 to March 2004, and SCIRUS³) and subscription access databases (EMBASE⁴ from 1994 to March 2004, and Cochrane Library⁵ and the Web of Science⁶).

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3 SCIRUS, http://www.scirus.com
4 EMBASE, http://www.embase.com/
5 Cochrane library, http://www.cochrane.org/index0.htm
6 Web of Science, http://wos.mimas.ac.uk/
The following search terms were used: (dengue) AND (IgM) AND (diagnosis) OR (sensitivity) OR (specificity) OR (monitoring) OR (ROC*) OR (reference value*) OR (diagnos*).

Study selection using standardized quality assessment criteria. Full-text articles of the selected studies were assessed by two researchers using standardized criteria to determine whether they were of sufficient quality to be included in the final meta-analysis. Standard quality assessment criteria were employed to assist in the selection process using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) tool (Whiting et al., 2003).

Data extraction. Data were extracted from the accepted studies by two researchers who performed quality assessment. The data were recorded on a standard form that included citation information, sample population characteristics, assay characteristics and results. Only IgM results for acute (admission) collections against a valid reference comparator were extracted from the original study results.

Analysis and data synthesis. Recommended methods for the meta-analysis of results of diagnostic tests were used to assess the selected studies (Deeks, 2001; Deville et al., 2002). Individual and pooled analysis of the extracted study data was performed using the statistical software program STATA™ version 7.0 (STATA Corporation, USA) using the METAN statistical routine (Bradburn et al., 2004). Summary receiver operator characteristic (SROC) curves were constructed using the MetaTest program version 0.6 (Lau, 1997).

Individual study data analysis. A 2 × 2 table was constructed in which a “gold standard” result or reference standard result was compared with the index test (the assay under investigation) to define true positive (Tp), false positive (Fp), false negative (Fn) and true negative (Tn) values. Using the 2 × 2 table values, the standard diagnostic accuracy indices of sensitivity, specificity, positive likelihood ratio (+LR), negative likelihood ratio (-LR) and diagnostic odds ratio (DOR) were calculated. A DOR of greater than 1 indicates that the odds ratio for a positive test result is greater for patients with the condition, and a DOR of less than 1 indicates that the odds ratio for a positive test result is greater for patients without the condition (Zhou et al., 2001). The +LR and the -LR describe how informative is the positive or negative test result (Habbema et al., 2002). By definition, a test with a +LR and -LR of 1.0 is worthless, and a +LR of 2.0 and -LR of 0.5 are the minimum useful values (Gill et al., 2003). A +LR of less than 10 and -LR of less than 0.1 generate large and often conclusive shifts from pre-test to post-test probability, thereby demonstrating high accuracy (Pai et al., 2003). A diagnostic assay with good levels of positive and negative discrimination will have a +LR of greater than 10 and the -LR should be less than 0.1.

Statistical pooling. Pooling of the individual study results was used to generate an overall estimate of diagnostic accuracy. Because of problems in pooling heterogeneous data, chi-squared statistics were calculated before pooling to detect significant heterogeneity (P < 0.1) overall and between subgroups. If heterogeneity was not significant then a fixed model (Mantel & Haenszel, 1959) was used to calculate results and, when significant, the random effects model was used (DerSimonian & Laird, 1986).

Results and discussion

Search results. The search of electronic databases identified a total of 302 studies. After scanning the abstracts, a total of 27 studies were retained for quality assessment using the full text. A further nine studies were identified by reading reference lists and searching journals by hand.

Quality. After quality assessment, 11 studies were accepted for inclusion in the
final meta-analysis and 25 studies were rejected. A full explanation of the reasons for rejection is available on request. The most common reason (56%) for the rejection of studies was the application of inappropriate reference assays or partial verification of the study sample by the reference assay.

Data extraction and description of studies. Details of data extracted from individual studies are presented in Table 6. All studies used the Panbio ICT as the index test. Five different “gold standard/reference tests were used to determine the diagnostic accuracy of the ICT (Panbio Duo ELISA, Panbio IgM ELISA, MRL IgM ELISA, AFRIMS MAC-ELISA and HAI). Sample recruitment used either prospective cohort (suspected dengue infections; five studies) or case–control methodologies (six studies). The timing of sample collections was an important consideration in the determination of diagnostic accuracy, as acute-phase or hospital-admission samples only could be included for this analysis. Examination of the study results revealed that while hospital-admission samples were used in many of the studies, several used studies of late acute infection (more than 7 days after the onset of symptoms) in the assessment of index tests. Samples were therefore classified into “early acute” for hospital-admission samples and “late acute” for samples collected between 7 and 10 days after the onset of symptoms, but not late enough to be considered as convalescent samples (convalescent samples were not included in this analysis). Disease severity in the patient was considered during the data extraction process. For a subset of studies it was also possible to separate sample results into primary and secondary dengue infection on the basis of infection status. A number of studies assessed the ability of the ICT to classify primary (IgM-positive/IgG-negative), secondary (IgM-positive/IgG-positive) and negative (IgM-negative/IgG-negative) relative to HAI or MAC-ELISA results (Sang et al., 1998; Vaughn et al., 1998; Vajpayee et al., 2001; Kittigul & Suankeow, 2002; Chakravarti et al., 2003).

Individual and pooled study diagnostic accuracy results. Significant heterogeneity (P < 0.1) between the studies was evident in sensitivity, specificity, DOR, +LR and -LR chi-squared statistical results (Table 7), thereby reducing the validity of statistical pooling of individual study results. Pooled results for sensitivity and specificity of the dengue ICT demonstrated similar ranges of values for 95% confidence intervals (CI). The pooled sensitivity result was 0.86 (95% CI, 0.74–0.92) and the pooled specificity result was 0.88 (95% CI, 0.78–0.94). The SROC curve analysis (Table 8) gave a weighted result for the area-under-the-curve (AUC) of 0.9105. The pooled DOR was 91 (95% CI, 25–333), the pooled +LR was 7.2 (95% CI, 3.7–14.2) and the pooled -LR was 0.16 (95% CI, 0.08–0.32).

Subgroup analysis

Diagnostic capacity. The most compelling evidence that the dengue ICT has acceptable diagnostic accuracy was the subgroup analysis using the Panbio Duo ELISA as the reference assay, allowing the pooling of four studies to determine the accuracy of the dengue ICT (Table 8). The diagnostic accuracy of the Panbio Duo ELISA has been determined in previous studies (Sang et al., 1998; Porter et al., 1999; Cuzzubbo et al., 1999) after assessment against the HAI “gold standard” assay, with sensitivity and specificity results that ranged from 0.88 to 0.99 and 0.92 to 1.00, respectively. Pooled diagnostic accuracy results of the dengue ICT compared with the Panbio Duo ELISA gave high sensitivity (0.90, 95% CI, 0.81–0.94), specificity (0.86, 95% CI, 0.76–0.92) and DOR results (207; 95% CI, 63–674). The +LR (7.6; 95% CI, 4.9–12.0) demonstrated that a sample from a dengue patient had an eightfold higher chance of giving a positive result in a dengue ICT (‘ruling in’) compared with samples from patients without dengue infection. The -LR (0.08; 95% CI, 0.04–0.15) was less than 0.1, which demonstrates that the ICT was also
acceptable at ‘ruling out’ samples that were negative for dengue. SROC curve analysis of the dengue ICT compared with the Panbio Duo ELISA gave an AUC-weighted result of 0.98, which demonstrates a high degree of accuracy.

**Improved diagnostic capacity using “late acute” samples.** The timing of sample collection had a large influence on the diagnostic accuracy of the dengue ICT (Table 9). A clear improvement in diagnostic accuracy was demonstrated for the “late acute” (7–10 days after onset of symptoms) subgroup of samples when compared with the “early acute” (hospital-admission) subgroup with higher sensitivity (0.96 versus 0.69), DOR (600 versus 17), +LR (8.5 versus 4.9) and lower -LR results (0.03 versus 0.32). Further evidence is provided by the SROC curve analysis, which gave an “early acute” sample AUC-weighted result of 0.87 and “late acute” sample AUC-weighted result of 0.99.

**Capacity to detect primary and secondary infections with dengue.** There was evidence that the dengue ICT was able to detect primary and secondary dengue infection using the diagnostic criteria outlined by the manufacturer, although low sensitivity was demonstrated for both infection states. Sensitivity (0.71 versus 0.66) and DOR (72 versus 72) results were similar for both primary and secondary dengue samples. However, diagnostic capacity for the detection of primary infections by the dengue ICT was improved, as indicated by the +LR result (10.4 versus 4.8) and sensitivity (0.95 versus 0.76). This was also demonstrated in the SROC curve analysis of the results of studies of primary infection, which gave an AUC-weighted result of 0.95, while the result for studies of secondary infection was 0.91.

**Clinical implications and conclusions**
There is evidence from this meta-analysis that the dengue ICT is a useful diagnostic test; however, it must be stressed that the assay must be used bearing in mind its limitations.

There is clear evidence that the timing of sample collection is very important for obtaining results with a high level of diagnostic confidence. Because the dengue ICT is a point-of-care style of assay, it may be expected that this assay will be used when a patient presents with an acute febrile illness to a hospital outpatients’ department or a doctor’s surgery. The onset of the symptoms of dengue infection usually occurs 5 days after infection, which may be too early for the detection of dengue-specific IgM antibodies (Gubler et al., 1998). This fact is well illustrated by the comparison of diagnostic accuracy indices for early acute and late acute infection samples; significantly higher results are recorded for samples collected 7–10 days after the onset of symptoms than for hospital-admission samples. It should be noted that none of the studies specified the number of days after onset of symptoms for the hospital-admission samples. An estimate that hospital-admission samples were collected 4–6 days after onset of symptoms would help to explain the dramatic differences in diagnostic ability of the ICT between the early and late acute samples. Therefore, the timing of sample collection is probably the most important aspect in the accurate diagnosis of dengue infection when using the dengue ICT. As with all infectious diseases, a second serum sample should be collected 3–4 days after the collection of a negative acute sample.

Assessment of the diagnostic ability of the dengue ICT to detect primary and secondary infections with dengue determined that, while sensitivity was suboptimal, the assay possessed sufficient diagnostic capacity, if interpreted with caution. Geographical setting must, however, be taken into account as many locations where dengue is endemic will have high pre-test odds for the presence of anti-dengue IgG or previous infection with another flavivirus; in such circumstances the result must be interpreted with caution.
TABLE 6. Details of data extracted from the selected studies

<table>
<thead>
<tr>
<th>Study author</th>
<th>Index assay</th>
<th>Reference assay</th>
<th>Study design</th>
<th>Disease severity</th>
<th>Sample collection timing or group</th>
<th>Dengue infection status</th>
<th>Tp</th>
<th>Fp</th>
<th>Fn</th>
<th>Tn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lam &amp; Devine (1998)</td>
<td>Panbio ICT</td>
<td>Panbio Duo ELISA</td>
<td>Case–control</td>
<td>NS</td>
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<td>Primary</td>
<td>7</td>
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<td></td>
<td></td>
<td></td>
<td>NS</td>
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<td>Secondary</td>
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<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
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<td>Early acute</td>
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<td>Dengue negatives</td>
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<td>12</td>
<td>95</td>
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<td>Panbio IgM ELISA</td>
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<td>Negative control group</td>
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<td>Panbio Duo ELISA</td>
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<td>Panbio IgM ELISA</td>
<td>Case–control</td>
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<td>MRL IgM ELISA</td>
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<td>Early acute</td>
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<td>DF/DHF/DSS</td>
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<td>Early acute</td>
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<td>Panbio Duo ELISA</td>
<td>Fever</td>
<td>DF</td>
<td>Late acute (7–10 days)</td>
<td>Primary</td>
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<td></td>
<td>DF</td>
<td>Late acute (7–10 days)</td>
<td>Secondary</td>
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<td>Late acute (7–10 days)</td>
<td>Dengue negatives</td>
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<td>Fever</td>
<td>NS</td>
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<td>Secondary</td>
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<td>Panbio ICT</td>
<td>AFRIMS MAC-ELISA</td>
<td>Case–control</td>
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<td>HAI</td>
<td>Case–control</td>
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<td>Primary</td>
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</tr>
</tbody>
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DF, Dengue fever; DHF, Dengue haemorrhagic fever; DSS, Dengue shock syndrome; Fn, False negative; Fp, False positive; HAI, Haemagglutination inhibition; NS, Not stated; Tn, True negative; Tp, True positive.
**TABLE 7.** Subgroup analysis, categorized by reference assay, to calculate individual and overall accuracy of results of ICTs for the detection of IgM antibodies to dengue

<table>
<thead>
<tr>
<th>Reference assay</th>
<th>No. of studies</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>DOR (95% CI)</th>
<th>+LR (95% CI)</th>
<th>-LR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Panbio Duo ELISA</td>
<td>4</td>
<td>0.92* (0.80–0.97)</td>
<td>0.91 (0.70–0.98)</td>
<td>206 (63–674)</td>
<td>7.7 (4.9–12.0)</td>
<td>0.08 (0.04–0.15)</td>
</tr>
<tr>
<td>Panbio IgM ELISA</td>
<td>2</td>
<td>0.87 (0.77–0.93)</td>
<td>0.95 (0.84–0.99)</td>
<td>160 (30–844)</td>
<td>41.8 (5.7–304.1)</td>
<td>0.21 (0.13–0.34)</td>
</tr>
<tr>
<td>MRL IgM ELISA</td>
<td>1</td>
<td>1.00 (0.95–1.00)</td>
<td>0.86 (0.56–0.97)</td>
<td>1287 (60–27 873)</td>
<td>9.1 (2.9–29.9)</td>
<td>0.01 (0.00–0.11)</td>
</tr>
<tr>
<td>AFRIMS MAC-ELISA</td>
<td>1</td>
<td>0.64 (0.52–0.74)</td>
<td>0.72 (0.56–0.83)</td>
<td>5 (2–10)</td>
<td>2.3 (1.4–3.7)</td>
<td>0.50 (0.35–0.71)</td>
</tr>
<tr>
<td>HAI</td>
<td>3</td>
<td>0.76* (0.52–0.90)</td>
<td>0.84 (0.53–0.96)</td>
<td>26 (12–57)</td>
<td>5.8* (1.2–28.2)</td>
<td>0.31 (0.23–0.44)</td>
</tr>
</tbody>
</table>

* Denotes significant heterogeneity (P < 0.1) and therefore the random effects model was used to calculate the results.

DOR, Diagnostic odds ratio; ELISA, Enzyme-linked immunosorbent assay; MAC-ELISA, IgM antibody capture ELISA; HAI, Haemagglutination inhibition.

**TABLE 8.** Subgroup analysis, categorized by sample timing, to calculate individual and overall accuracy of results of ICTs for the detection of IgM antibodies to dengue

<table>
<thead>
<tr>
<th>Sample timing</th>
<th>No. of studies</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>DOR (95% CI)</th>
<th>+LR (95% CI)</th>
<th>-LR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early acute</td>
<td>7</td>
<td>0.73* (0.62–0.75)</td>
<td>0.88* (0.74–0.95)</td>
<td>33* (10–114)</td>
<td>6.3* (2.8–13.9)</td>
<td>0.33* (0.21–0.52)</td>
</tr>
<tr>
<td>Late acute</td>
<td>4</td>
<td>0.96* (0.90–0.98)</td>
<td>0.90* (0.65–0.98)</td>
<td>600 (11–28)</td>
<td>8.5 (5.1–14.3)</td>
<td>0.03 (0.01–0.07)</td>
</tr>
</tbody>
</table>

* Denotes significant heterogeneity (P < 0.1) and therefore the random effects model was used to calculate the results.

CI, Confidence interval; DOR, Diagnostic odds ratio; ICTs, immunochromatographic tests; LR, Likelihood ratio.

**TABLE 9.** Subgroup analysis, categorized by infection status, to calculate individual and overall accuracy of results of ICTs to determine primary or secondary infection

<table>
<thead>
<tr>
<th>Infection status</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>DOR (95% CI)</th>
<th>+LR (95% CI)</th>
<th>-LR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>0.71* (0.37–0.91)</td>
<td>0.95* (0.76–0.99)</td>
<td>72 (21–290)</td>
<td>10.4 (5.0–21.7)</td>
<td>0.30 (0.20–0.45)</td>
</tr>
<tr>
<td>Secondary</td>
<td>0.66 (0.56–0.75)</td>
<td>0.76 (0.67–0.83)</td>
<td>72* (4–1257)</td>
<td>4.8* (1.8–12.4)</td>
<td>0.14* (0.04–0.56)</td>
</tr>
</tbody>
</table>

* Denotes significant heterogeneity (P < 0.1) and therefore the random effects model was used to calculate the results.

CI, Confidence interval; DOR, Diagnostic odds ratio; ICTs, immunochromatographic tests; LR, Likelihood ratio.
References


Diagnostic needs for dengue
DETECTION OF NS1 FROM DENGUE VIRUS: BASIS FOR EARLY DIAGNOSIS AND A PROGNOSTIC MARKER OF DISEASE PROGRESSION (1)

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The flavivirus NS1 is a 46–50 kilodalton glycoprotein which is expressed in both membrane-associated (mNS1) and secreted (sNS1) forms and possesses both group-specific and type-specific determinants. Unusually for a viral glycoprotein, it does not form part of the virion structure but is expressed on the surface of infected cells as well as being secreted. NS1 is initially translocated into the endoplasmic reticulum (ER) via a hydrophobic signal sequence encoded in the C-terminal region of E, where it rapidly dimerizes. sNS1 undergoes further oligomerization before secretion as it has been shown to be hexameric. While the function of NS1 is yet to be fully defined, preliminary evidence has shown it to be involved in viral RNA replication.

NS1 was first described as a soluble complement fixing (SCF) antigen in infected cell cultures. The identity of SCF as the viral-encoded 46 kilodalton glycoprotein gp46 was established; it was later re-named NS1 following the sequencing of the yellow fever virus genome. The flavivirus NS1 has been recognized as an important immunogen in infections with antibodies elicited to this protein, and has been shown to play a role in protection against disease. However, potential roles in pathogenesis for both NS1 and antibodies to NS1 have also been proposed based on the finding that both can bind to, and potentially activate, endothelial cells. Given that circulating immune complexes and complement activation are also integral features of the more severe forms of disease, DHF and DSS, the anamnestic anti-NS1 antibody response seen in secondary infections coupled with a high level of secreted NS1 in the sera of these patients suggests that they may also play a significant role in pathogenesis through immune complex formation.

In order to examine the potential of NS1 as a diagnostic marker of dengue infection as well as to more clearly understand its role in pathogenesis, we developed the first capture ELISA for the detection of the flavivirus NS1 in patient sera (Hilditch et al, 1991; Young et al, 2000). The assay employs rabbit polyclonal and monoclonal antibodies as the capture and detection antibodies respectively. Immuno-affinity purified NS1 derived from dengue 2 virus infected cells has been used as a standard to establish a detection sensitivity of approximately 4ng/ml for an assay employing monoclonal antibodies recognizing a dengue 2 serotype specific epitope (see below). A number of serotype cross-reactive monoclonal antibodies were also shown to be suitable probes for the detection of NS1 expressed by the remaining three dengue virus serotypes.

Examination of clinical samples demonstrated that the assay was able to detect NS1 with minimal interference from serum components at the test dilutions routinely used, suggesting that it could form the basis of a useful additional diagnostic test for dengue virus infection. Furthermore, our earlier studies suggested that quantitation of NS1 levels in patient sera may prove to be a valuable surrogate marker for viremia. This has been subsequently confirmed with an analysis we performed of sNS1 plasma levels measured daily in 32 children with dengue-2 virus infections participating in a prospective, hospital-based study in Thailand (in collaboration with Dan Libraty, Armed Forces Research Institute of Medical Sciences
Plasma sNS1 levels were indeed found to correlate with viremia levels (Libraty et al., 2002). Surprisingly high levels of NS1, as much as 15 µg/ml, were found in acute phase sera taken from some of the patients experiencing serologically confirmed dengue 2 virus secondary infections. Lower levels of NS1 have also been detected in primary infected patients. The presence of such high levels of secreted NS1 in the sera of patients experiencing secondary dengue virus infections, and in the context of an anamnestic antibody response, suggests that NS1 may contribute significantly to the formation of the circulating immune complexes that are suspected to play an important role in the pathogenesis of severe dengue disease (as indicated above).

It is possible that NS1 may also be playing a more direct role in pathogenesis given the ability of this protein to bind to endothelial cells. Our studies have supported the hypothesis of a direct involvement by NS1 in disease progression as levels were found to be higher in patients with DHF compared to DF. An elevated plasma sNS1 level (> 600 ng/ml) within 72 h of illness onset identified patients at risk for developing DHF, suggesting that the detection of NS1 in patients prior to the onset of DHF/DSS would be an excellent prognostic marker for severe disease progression.

In summary, we have shown that secreted NS1 in patient sera may provide an ideal diagnostic marker for dengue virus infection during the early phase of infection when antibody levels are not detectable. Furthermore, a direct correlation between high levels of NS1 early in infection and subsequent severe disease indicates that it may also serve as a prognostic marker of disease progression.

**FIGURE 5.** Dengue NS1 capture ELISA. This ELISA plate shows a 1:10 screen of patient serum samples. Each sample is in triplicate and the plate also has a duplicate titration of an NS1 standard (diluted in PBS containing 1:10 NHS) and -ve controls.
References
DETECTION OF NS1 FROM DENGUE VIRUS: BASIS FOR EARLY DIAGNOSIS AND A PROGNOSTIC MARKER OF DISEASE PROGRESSION (2)

Marie Flamand, Sophie Alcon-LePoder, Marie-Thérèse Drouet, Peggy Sivard
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Nonstructural glycoprotein NS1 of flaviviruses is essential for virus viability. During host-cell infection in vitro, NS1 is found associated with the cell surface and with intracellular organelles, where it may participate in the viral replication process. We have shown that NS1 is eventually released as a soluble hexamer from infected mammalian cells into the extracellular fluid, but not from vector-derived mosquito cells.

To investigate the biological relevance of NS1 secretion in vivo, we searched for circulating NS1 in the serum of patients infected with DENV-1 on different days after the onset of fever. For this purpose, we developed an ELISA test based on antigen capture (capture-ELISA) and using NS1-specific antibodies. We found that NS1 was present in the blood stream of a large percentage of patients tested during the acute phase of the disease. NS1 could be detected from day 1 after the first fever (set at day 0) until day 6 in most if not all patients (Alcon et al., 2002), while none of the convalescent sera were positive for NS1 antigen, presumably because all the protein becomes complexed to antibodies or is no longer produced by infected host cells.

Using DEN cross-reactive monoclonal antibodies, we were able to process a large panel of sera, recovered from within the Pasteur Institute Network, collected during epidemics involving DENV-1 and -2 in French Guiana, 1996–1997 (in collaboration with A. Talarmin), DENV-3 in Guadeloupe, 2000 2001 (in collaboration with J.-L. Cartel) and DENV-4 in Viet Nam, 2001 (in collaboration with Vu Thi Que Huong). Between days 1 and 6 after the onset of fever, 87%, 70%, 77% and 60% of the sera, respectively, were found to give a positive result with our NS1-capture ELISA. Using a similar approach, NS1 of West Nile virus (WNV) could be detected in experimental infections in sera from mice (collaboration with P. Desprès, Institut Pasteur) and chickens (collaboration with C. Céianu, Romania). With regard to natural infections, a few domestic fowls included in an epidemiological survey programme carried out in 1999 in Romania, as well as 17% of Camargue horses that developed neurological symptoms during the 2000 WNV epizootic in France (collaboration with S. Zientara, Agence française de sécurité sanitaire des aliments, and H. Zeller, Centres Nationaux de Référence, Institut Pasteur, France) were also shown to be positive for NS1. Most importantly, the protein was detected in 40% of sera from patients showing clinical signs of WNV-associated encephalitis (1996 outbreak, Romania) but not in the corresponding cerebrospinal fluids.

In conclusion, detection of NS1 antigen from flavivirus in sera may represent an interesting diagnostic approach to provide evidence for ongoing infections, and should also be considered for use in the case of asymptomatic infections associated with a risk of blood transfusion-related transmission. Finally, if used in a systematic screening programme, NS1 capture-ELISA may facilitate the improvement of epidemiological surveys of flavivirus circulation.

Reference
Alcon S et al. (2002). Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. Journal of Clinical Microbiology, 40:376–381.
DETECTION OF ANTIBODIES TO DENGUE: NEUTRALIZATION

Scott B. Halstead
Director of Research, Pediatric Dengue Vaccine Initiative, Bethesda, Maryland, USA

What role do antibodies play in dengue in humans?
As illustrated by clinical disease observed in human infants, antibodies play a dual role. Initially, dengue antibodies acquired transplacentally in utero are protective, but degrade over time and are associated with enhanced disease seen during the second half of the first year of life (Figure 6). This can best be understood (Figure 7) as demonstrating the neutralizing effect of high concentrations of maternal antibodies and the infection enhancement phenomenon at the time when antibody concentrations fall below neutralization thresholds, but are biologically active in promoting antibody-dependent enhancement (Halstead, 1970; Halstead et al., 2002; Halstead, 2003).

It should be noted that only IgG1 antibodies cross the placenta. Such antibodies—but not T memory cells—are sufficient to control infection outcome, either by prevention or enhancement.

How do antibodies neutralize the virus?
• The consensus of modern researchers is that neutralization is a direct consequence of attachment of antibody to the virus, and is not caused by aggregation of viruses (Burton, 2001; Burton et al., 2001).
• Under conditions of antibody excess, the kinetics of neutralization are first-order (Figure 8).
• Neutralization is independent of the assay system (Dulbecco et al., 1956).
• Virus–antibody complexes are stable.
• The rate of neutralization is dependent upon temperature (Figure 9).

FIGURE 6. Age distribution of infants with dengue hemorrhagic fever admitted to Bangkok Children’s Hospital, Thailand, in selected years, 1987–1998
**FIGURE 7.** Schema illustrating the relationship between catabolism of maternal dengue antibodies and the incidence of dengue hemorrhagic fever in infants during a primary dengue infection.

From Halstead et al. (2002)

**FIGURE 8.** First order neutralization kinetics

Reprinted from: Dulbecco RM, Vogt RM and Strickland BG, 1956, with permission from Elsevier.
**FIGURE 9.** Effect of temperature on neutralization kinetics

Temperature: 1 = 37 °C; 2 = 20 °C; 3 = 4 °C

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**FIGURE 10.** Effect of serum concentrations on neutralization kinetics

Dilution of serum: 1 = 1:10; 2 = 1:100; 3 = 1:1000

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• The rate of neutralization is dependent upon concentration of antibody (Figure 10).
• Virus particles are capable of combining with more than one antibody molecule (Burton, 2001).
• Each virus particle has $n$ critical sites on its surface. The larger the virus, the more critical sites.
• When $n$ sites are covered, the virus is neutralized (Della-Porta & Westaway, 1977).

**How are neutralizing antibodies measured?**

**In animals**

*In mice.* Using intracerebral or intraperitoneal routes of inoculation, with death as an end-point. Now seldom used is the serum constant, virus dilution method, which results in the calculation of a log neutralization index. This assay may measure avidity as well as concentration of neutralizing antibody (Halstead & Russ, 1962).

*In monkeys.* Using the intradermal or subcutaneous route of inoculation, with viraemia as the end-point. This model is almost never used to test passively transferred antibodies or mixtures of serum and virus, although it is probably highly relevant biologically. Monkeys can be used as a challenge model to test the protection afforded by actively acquired antibodies (Halstead & Palumbo, 1973; Halstead et al., 1973).

**In tissue culture**

*The plaque-reduction or the fluorescent focus reduction methods* (virus constant versus dilutions of antibody) (Russell et al., 1967; Okuno et al., 1978) are used by most workers. Crucial variables that must be controlled include:
• plaque-overlap phenomenon—when plaque crowding occurs, the countable number of plaques does not correspond to input dose (in plaque-forming units). Plaque overlap may reduce the plaque count by three- to fourfold, depressing neutralizing antibody titres;
• usefulness of the ‘accessory factor’ found in unheated serum and that is thought to be human complement;
• use of the 50% plaque-reduction end-point calculated using log probit graph paper or computer program;
• three widely-spaced (10-fold) dilutions may be optimal for identifying the 50% plaque-reduction end-point. Further dilution may result in inaccurate titres when using computer programs.

**References**


Halstead SB et al. (2002). Infant dengue hemorrhagic fever: research opportunities ignored. Emerging Infectious Diseases, 8(12):1474–1479.


ANTIBODIES AGAINST DENGUE VIRUSES: PROBLEMS OF INTERPRETATION WHEN MORE THAN ONE FLAVIVIRUS CO-CIRCULATES IN A COMMUNITY

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Universiti Malaysia Sarawak, Sarawak, Malaysia.

Introduction
In many developing countries, multiple infectious agents co-circulate in a community. This presentation aimed to emphasize the fact that, because flaviviruses have shared epitopes, cross-reactive antibody responses may develop. This can lead to problems with the interpretation of antibody reactivity if serological tests do not take into consideration the universe of flaviviruses that may be circulating in a community. For example, in Sarawak in Malaysian Borneo, Japanese encephalitis is a major problem and dengue virus also circulates both in urban and rural areas. Three main points were made in this presentation:

Flaviviruses have shared epitopes and elicit cross-reactive antibodies, as measured by enzyme immunoassays. Figure 11 provides typical examples of the results of ELISAs carried out on serial bleeds of patients with dengue or Japanese encephalitis infections.

Panels A and B show the IgM and IgG responses, respectively, of an 8-year-old boy with dengue haemorrhagic fever, who had a high level of IgM against dengue virus antigens at day 4 after the onset of fever, the level staying high for 6 weeks. In

**FIGURE 11.** Examples of IgM and IgG responses in patients with dengue or Japanese encephalitis

DEN, Dengue; JEV, Japanese encephalitis virus; OD, optical density
the acute phase, this patient also had cross-reacting IgM antibodies against JEV, but the level of cross-reacting antibodies waned with time and were not detectable by 6 weeks. The levels of IgG against dengue and JEV antigens were high in the acute phase and remained high throughout the 6 weeks. This is an example of a patient with a secondary dengue infection. Neutralization tests (not shown) suggested that the patient had also previously experienced a JEV infection.

Panels C and D show the IgM and IgG responses, respectively, of a 5-year-old girl with Japanese encephalitis. The antibody response to both JEV and dengue antigens rises over time. The optical density (OD) readings were consistently higher for JEV than for dengue, and neutralization tests (not shown) were positive for JEV and not for dengue. The cerebrospinal fluid taken on day 8 gave a positive result for IgM for JEV but not for dengue.

This figure illustrates the importance of testing for both dengue and JEV antigens in parallel (on the same plate) in order to distinguish between infection with JEV and dengue, if neutralization tests are not done routinely.

Do a complete virological workout in order not to miss multiple infections. Another important issue in the diagnosis of infectious diseases in developing countries, which is related to the co-circulation of many infectious agents, is that in some instances a patient may be infected with more than one agent, either concurrently or in rapid succession.

One example which illustrates this problem is shown in Figure 12. A 10-month-old girl presented with dengue haemorrhagic fever and central nervous system manifestations during the peak of an outbreak of hand, foot and mouth disease in May 2000. Paired serum taken at admission and again 4 days later showed a rising IgM antibody response to dengue antigens but not to JEV antigens. The cerebrospinal fluid was negative for IgM against both dengue and JEV antigens. The same cerebrospinal fluid yielded an enterovirus in rhabdomyosarcoma cells and this was identified as human enterovirus 71 (HEV71), which was the main etiological agent

**FIGURE 12.** Dual infection: dengue haemorrhagic fever with neurological involvement

CSF, Cerebrospinal fluid; DEN, Dengue; JEV, Japanese encephalitis virus; OD, Optical density

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EV71 was isolated from CSF
responsible for the hand, foot and mouth disease outbreak occurring concurrently (Cardosa et al., 2003).

Another example (not shown here) is the isolation of Coxsackievirus A 16 from the vesicles of a child with hand, foot and mouth disease and encephalitis in late 2000. Serum and cerebrospinal fluid were positive for IgM against JEV.

Antibodies against prM distinguish between previous JEV and dengue infection. Figure 13 is a modification of data presented in Cardosa et al. (2002) and shows that it is likely that antibodies to the envelope pre-membrane glycoprotein prM may be useful as a screening tool in serological surveys to determine the prevalence of JEV and dengue infection. Development of ELISA tests with different flavivirus prM antigens would provide a faster, high-throughput alternative to neutralization tests. This would be useful for vaccine trials in areas where dengue co-circulates with other flaviviruses.

References
THE IGM-CAPTURE ELISA USING VLPS OR VIRUS-INFECTED MOUSE BRAIN ANTIGENS TO DETECT IGM ANTIBODY IN SERUM FROM PATIENTS WITH EVIDENCE OF FLAVIVIRAL INFECTION

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Introduction
Members of the genus Flavivirus have a genome of about 11 kB, comprising a single-stranded, positive-sense RNA that translates and encodes capsid (C), pre-membrane/membrane (prM/M), and envelope (E) structural proteins and seven nonstructural proteins (NS). During natural flavivirus infections, non-infectious VLPs are produced, in addition to infectious, mature virions (Russell et al., 1980). Flavivirus VLPs have structural and physiochemical properties that are similar to those of mature virus particles. VLPs have been characterized for several flaviviruses, including tick-borne encephalitis virus (Schalich et al., 1996), JEV (Hunt et al., 2001; Konishi et al., 1992), West Nile virus (WNV) (Davis et al., 2001), St Louis encephalitis virus (SLEV) (Purdy et al., 2004), DENV-2 (Chang al., 2003), DENV-1, -3 and -4 (Purdy & Chang, 2005), and Murray Valley encephalitis virus (Kroeger et al., 2002). Plasmid DNA containing an eukaryotic transcriptional unit consisting of the human cytomegalovirus immediate early gene promoter, Kozak consensus ribosomal binding sequence, the signal sequence derived from the carboxyl-terminus of the C protein of JEV, and the prM/M and E gene regions is sufficient for the production of VLPs. The transformation of tissue culture cells with plasmid DNA is therefore advantageous for antigen production, since these cells then secrete VLPs containing viral prM and E proteins with the same conformation and presentation of epitopes as those of virion particles.

The isolation and characterization of virus, detection of genomic sequence, viral-specific antigen(s), and dengue virus-specific antibodies are the most commonly employed methods for the diagnosis of infection (Shu & Huang, 2004). In the serodiagnosis of infection with dengue virus, detection of virus-specific IgM and IgG antibodies by ELISA is a simple method that facilitates the processing of numerous samples of serum. Differentiation between primary and secondary infections can be achieved by determining the ratio of IgM to IgG in acute-phase sera (Innis et al., 1989; Shu et al., 2003). Demonstration of a fourfold or greater increase in IgM and/or IgG antibody titres between paired samples of sera collected during the acute and convalescent phases is a reliable method for determination of a recent infection. IgM antibodies are generally less cross-reactive than IgG in primary infections; the serotype having the highest IgM titre often corresponds to the virus responsible for current infection. In addition to increasing the potential risk of severe disease caused by antibody-dependent enhancement following a secondary infection with dengue virus, the presence of cross-reactive antibodies makes the differential diagnosis of dengue virus infection difficult. Determination of primary versus secondary infection and serotyping of the most recently infecting dengue virus, especially in areas where multiple serotypes co-circulate, requires that both IgM and IgG ELISAs be conducted, or that paired serum samples be simultaneously tested in the same ELISA. However, definitive information about serum samples, such as the collection date, the date of onset of symptoms or patient travel and/or vaccination history, is often not readily available for clinical diagnostic laboratories.
The majority of ELISA formats described for the detection of DENV-specific antibody use virus-infected cell culture supernatants or suckling mouse brain (SMB) preparations as the serodiagnostic antigens. The VLP antigens are excellent alternatives to these antigens for this purpose; they are not infectious, do not require the use of live virus or hazardous chemicals for preparation, and can be easily concentrated from the tissue culture fluid of transiently-transformed cells or continuously-secreting, clonally-selected cell lines by ultracentrifugation. For detecting anti-flaviviral antibodies in human serum, ELISAs employing WNV, JEV and SLEV VLP antigens have comparable sensitivities and specificities to those using SMB antigens (Davis et al., 2001; Hunt et al., 2001; Purdy et al., 2004). We recently demonstrated that low cross-reactivity with anti-WNV IgM antibody makes the use of SLEV VLPs in MAC-ELISA screening of patient serum samples preferable to the use of antigens derived from SMB preparations (Purdy et al., 2004). In the study described here, we report the use of VLP antigens for four DENV serotypes in MAC-ELISA detection of antiflaviviral IgM antibody and compare these results with assays using the conventional virus-infected SMB antigens. Additionally, DENV-1 to -4, and previously developed JEV, WNV and SLEV VLP antigens were tested using serum panels from patients with evidence of infection with WNV, SLEV, JEV, Powassan virus (POWV) and La Crosse virus (LACV), in order to compare the performance of the assay using either VLPs or SMB antigens. COS-1 cell lines continuously secreting these DENV VLPs were clonally selected in order to establish a simple and standardized method for producing VLP antigens.

Materials and methods

Human serum. Serum specimens were obtained from the Diagnostic and Reference Laboratory (DRL), Arbovirus Diseases Branch (ADB), Division of Vector-Borne Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins CO, USA. Serum samples were randomly numbered and tested as a blind panel. Panels were assembled by selecting serum specimens collected between 1999 and 2003 that had neutralizing antibody titres to WNV (n = 10), SLEV (n = 5), POWV (n = 5), or LACV (n = 5), as determined by DRL using the “gold standard” 90% plaque-reduction neutralization test. The panel of sera (n = 27) with evidence of DENV infection was assembled by selecting specimens previously determined by DRL to be positive for anti-dengue IgM antibodies using a mixture of DENV-1, -2, -3 and -4 infected SMB antigens.

VLP antigen preparation. The DENV VLPs and normal Chinese hamster ovary cell culture antigen were concentrated and partially purified from clarified tissue culture medium of transiently-transformed Chinese hamster ovary cells by ultracentrifugation at 19 000 rpm for 8 to 16 hours in a Beckman Coulter Type 19 rotor at 4 °C. The pellet was resuspended in TN buffer (Tris, 50 mmol/l; NaCl, 100 mmol/l; pH 7.5) to one fiftieth of the original volume, aliquoted into 1 ml samples and stored at -70 °C until use. SLEV, WNV and JEV VLPs were prepared in a manner similar to that described above (Hunt et al., 2001; Purdy et al., 2004). Antigens were independently titrated against a positive-control serum sample using a two-fold dilution series and standardized by selecting a dilution that yielded an absorbance of 0.8–1.2 at 450 nm (A450). For preparation of the mixture of DENV-1 to -4 VLP antigens, an appropriate volume of each undiluted antigen was added at a ratio corresponding to its individual working dilution, as determined above. This antigen mixture was then titrated and standardized as above.

ELISA protocols. MAC-ELISAs were performed by DRL using the protocol described by Martin et al. (2000) with virus-infected SMB antigens. SMB antigens were titrated in the same manner against the same positive-control human serum
sample used for titration of VLPs, and standardized by selecting a dilution that yielded an A450 of 0.8–1.2.

Test validation and calculation of positive-to-negative ratio (P/N) values. Test validation and P/N values were determined according to the procedure of Martin et al. (2000), including sera that acted as internal positive and negative controls in each 96-well plate.

Statistical analysis. The sensitivity and specificity of a given assay is measured by the proportion of patients with evidence of infection who have a positive test result and the proportion of patients without evidence of infection or patients with clear evidence of having been infected by other arboviruses who have a negative test result, respectively. We defined the evidence of infection by using the following criteria to transform the numeric P/N ratio into five discrete categories: 0 = P/N of < 2 (definitive negative), 1 = P/N of ≥ 2 and < 3 (presumptive positive), 2 = P/N of ≥ 3 and < 4 (probable positive), 4 = P/N of ≥ 4 and < 5 (positive), and 5 = P/N of ≥ 5 (definitive positive). These transformed data were applied to the following analysis and interpretation.

The receiver operating characteristic (ROC) curve analysis, a plot of the sensitivity versus false-positive rate (100 - specificity), was applied to discriminate between the accuracy of tests employing VLPs or SMB antigens, using the MedCalc statistical package (Berkley, CA) (Shapiro, 1999). The performance of a diagnostic test, or the ability of a test to determine the evidence of infection in the target panel or no evidence of infection in the control panels, was evaluated using ROC curve analysis, and the discriminate accuracy was measured by the area under the ROC curve (AUC).

The likelihood ratio (LR) was calculated to indicate the predicted probability of the infection based on the different cut-off levels (P/N ratio). LR indicates the probability of a given test result among people with confirmed infection divided by the probability of that test result among people without the infection. Thus, the positive likelihood ratio (+LR) indicated the ratio between the probability of a positive test result given the presence of the infection and the probability of a positive test result given the absence of the infection. The magnitude of the LR of a given test result can be used to predict the likelihood of the evidence of infection.

Additionally, the public health impact of a new test can be inferred by the positive predictive value (PPV) and negative predictive value (NPV). PPV is the proportion of specimens with a positive test result that have evidence of infection. NPV is the proportion of specimens with a negative test result that do not have evidence of infection. The PPV and NPV are highly dependent on the proportion of the study population that does or does not have the infection in the study area. We applied the same cut-off criteria as used in the AUC calculation to define the evidence of infection or non-infection, respectively.

Results

Use of VLPs and SMB antigens in the MAC-ELISA. Arbovirus-infected human serum samples consisting of a WNV panel (n = 10), a SLEV panel (n = 5), a POWV panel (n = 5), a LACV panel (n = 5), and a DENV panel (n = 27) were obtained from DRL. WNV, SLEV and POWV are the most medically important flaviviruses in North America. LACV, a member of Bunyavirus of California serocomplex, was the most prominent arbovirus in the USA before the introduction of WNV in 1999. Panels were assayed for the presence of IgM antibody by MAC-ELISA. The assay results, expressed as the P/N ratio using VLPs or virus-infected SMB antigens, are summarized in Table 10.

Recombinant VLP antigens for the four DENV serotypes and for SLEV, WNV and
JEV were employed in MAC-ELISAs to determine virus-specific IgM as well as the extent of cross-reactive IgM antibodies in various human serum panels (Table 10). MAC-ELISAs employing the DENV-2 VLPs detected cross-reactive IgM antibody in one specimen in each of the SLEV and WNV serum panels (P/N of 3.6 and 3.0; data not shown). None of the other DENV VLP preparations detected cross-reactive antibodies in any non-DENV serum panel. As expected, VLP antigens for members of the JEV serogroup (JEV, WNV and SLEV) exhibited high degrees of cross-reactivity to antibodies produced against other members of this group. WNV and JEV VLPs detected cross-reactive IgM antibodies in two and five out of five SLEV-infected serum specimens, respectively. SLEV and JEV VLPs detected cross-reactive antibodies in two and nine out of ten serum specimens in the WNV panel, respectively. None of the seven VLP antigens detected the existence of cross-reactive antibodies in either the POWV or LACV panels. When compared with the corresponding VLP antigen, SMB antigens exhibited less specificity. The mixture of virus-infected SMB antigens for the four DENV serotypes (the current antigen mixture used by ADB-DRL in screening serum specimens for anti-dengue antibody) detected cross-reactivity in panels for SLEV (four out of five specimens; P/N values, ≥ 3.0; range, 4.0–14.3; average, 7.8; data not shown), WNV (eight out of ten specimens; P/N values, ≥ 3.0; range, 4.2–12.0; average, 6.8; data not shown), and POWV (two out of five specimens; P/N values, ≥ 3.0; range, 3.1–3.8; average, 3.5; data not shown).

The DENV VLPs were then applied to the DENV panel (n = 27) in the MAC-ELISA for IgM antibody detection. The P/N ratio for this DENV panel was determined previously by ADB-DRL employing the mixture of DENV-1 to -4 infected

### TABLE 10. The IgM-capture ELISA for detecting cross-reactive antibodies in human serum specimens with evidence of various arboviral infection

<table>
<thead>
<tr>
<th>Panel</th>
<th>VLP</th>
<th>SMB antigen</th>
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<tbody>
<tr>
<td></td>
<td>DENV-1–4</td>
<td>DENV-1</td>
</tr>
<tr>
<td>SLEV panel (5)</td>
<td>0*</td>
<td>0</td>
</tr>
<tr>
<td>WNV panel (10)</td>
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<td>0</td>
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<tr>
<td>POWV panel (5)</td>
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<td>0</td>
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<tr>
<td>LACV panel (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DENV panel (24)</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Unknown panel (3)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Negative control panel (3)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

DENV, Dengue virus; JEV, Japanese encephalitis virus; LACV, La Crosse encephalitis virus; ND, Not done; POWV, Powassan encephalitis virus; SLEV, St Louis encephalitis virus; SMB, Suckling mouse brain; WNV, West Nile virus; VLP, Virus-like particle

* Number of positive specimens, defined as having a P/N ratio of ≥ 3 when tested against VLP or SMB.
SMB antigens (P/N values, ≥ 3.0; range, 3.1–33.0; average, 9.3; data not shown). The mixture of DENV-1 to -4 VLP antigens detected IgM antibody in 21 out of 27 samples (P/N values, ≥ 3.0; range, 4.1–14.4; average, 7.1; data not shown). The VLP mixture did not detect IgM in 6 out of 27 positive specimens; however, three additional serum specimens (data not shown) tested positive with DENV-1, -2 or -4 VLP antigens. Three remaining specimens, reported as positive by ADB-DRL, gave completely negative results using all seven VLP antigens (SLEV, WNV, JEV, and DENV-1 to -4). Unfortunately, owing to insufficient volume of serum we were not able to perform the plaque-reduction neutralizing assay to confirm the presence of viral neutralizing antibodies. Thus, these three serum specimens were grouped as the unknown panel (Table 10), and excluded from further analysis.

Influence of VLPs and SMB antigens on assay performance measured by comparative ROC analysis. On the basis of calculation of the best cut-off of P/N ratio, we transformed the numerical P/N ratio into five discrete categories: 0 = P/N < 2 (definitive negative), 1 = P/N ≥ 2 and < 3 (presumptive positive), 2 = P/N ≥ 3 and < 4 (probable positive), 4 = P/N ≥ 4 and < 5 (positive), and 5 = P/N ≥ 5 (definitive positive). We then used this transformed data to compare the influence of VLPs and SMB antigen on overall assay performance and to determine whether there was any statistically significant difference between them in terms of sensitivity and specificity (Table 11). The P/N < 3 or ≥ 3 was used in classification of a given specimen as giving a negative or positive result, respectively, in the assay, and to define the evidence of infection (target panel) or non-infection (control panels), respectively. The overall performance of the MAC-ELISA using VLPs was significantly better than the MAC-ELISA using SMB antigens as determined in pair-wise comparison. The AUC was 0.99 (95% CI, 0.91–1.00) and 0.83 (95% CI, 0.70–0.92) for the mixture of all four DENV VLPs and SMB antigens, respectively, with P = 0.02. The AUC was 1.00 (95% CI, 0.84–1.0) and 0.94 (95% CI, 0.75–0.99) for SLEV VLP and SMB antigens, respectively, with P = 0.44. The WNV VLPs and SMB antigen pair was statistically different. The AUC was 0.96 (95% CI, 0.78–0.99) and 0.85 (95% CI, 0.64–0.96) for WNV VLP and SMB, respectively, with P = 0.05.

**Determination of the status of infection and seroprevalence.** The positive likelihood ratio (+LR) can be used to predict the evidence of infection for an individual based

<table>
<thead>
<tr>
<th>Table 11. The influence of VLPs or SMB antigens on the performance of MAC-ELISA to distinguish the infection serum panel (target panel) versus panels of serum infected with other arboviruses (control panels)</th>
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<tbody>
<tr>
<td><strong>Serum panel</strong></td>
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<td><strong>Target panel</strong></td>
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<tr>
<td>DENV</td>
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<td>SLEV</td>
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<td>WNV</td>
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DENV, Dengue virus; SLEV, St Louis encephalitis virus; SMB, Suckling mouse brain; WNV, West Nile virus; JEV, Japanese encephalitis virus; POWV, Powassan encephalitis virus; LACV, La Crosse encephalitis virus; VLP, Virus-like particle

* P = 0.02
** P = 0.05
on the P/N ratio reading of the assay from the patient's serum specimen. The +LR is influenced by the best cut-off of P/N of the assay (Table 12). The P/N ≥ 3 was used as an example to define the evidence of infection in the individual's serum specimen by the MAC-ELISA. The +LR was 20, 7.5 and ∞ for SLEV, WNV and DENV infection, respectively, when the respective SLEV, WNV and the mixture of DENV VLPs was used; however, the +LR was 3.4, 4.4, and 12.5 when using the respective SMB antigens. Use of VLPs thus significantly improved the predictive value of determining the evidence of infection compared with use of SMB antigens.

In addition, when the P/N ≥ 3 was used to define the evidence of infection (the current criteria used by DRL) and applied to a large scale sero-epidemiological study, the SLEV and WNV VLP also had a higher PPV (71.4% and 83.3%, respectively) and NPV (100% and 100%, respectively) than the PPV (41.7% and 63.6%) and the NPV (100% and 78.6%) for use of SMB antigens of SLEV and WNV, respectively. The mixture of DENV VLPs had a PPV of 100% and an NPV of 81.3% compared with the mixture of DENV SMB antigens (92.3% PPV and 100% NPV) (Table 12).

Discussion
Antigens secreted as VLPs by cloned cell lines are easily collected from the culture media, concentrated by polyethylene glycol or by ultracentrifugation, and lyophilized for long term storage. Such preparations function well in both MAC-ELISA and IgG-ELISA formats (Davis et al. 2001; Hunt et al., 2001; Purdy et al., 2004). The results of the current study showed that a higher specificity was obtained when VLPs were used in the MAC-ELISA than when SMB antigens were used, while simultaneously showing a similar degree of sensitivity, as seen in a previous study with DENV (Nawa et al., 2000).

The higher specificity obtained when using VLPs in the MAC-ELISA could be due to the better quality and higher purity of VLPs compared with SMB antigens. Viral infectivity in the SMB preparation is eliminated by chemical inactivation and the lipid components in the mouse brain are removed by acetone extraction procedures. There is no additional purification procedure involved in preparing SMB antigens. Conversely, VLPs are prepared from cell culture fluids by ultracentrifugation, which concentrates particulates or protein aggregates.

| Table 12. Influence of VLPs and SMB antigens on the MAC-ELISA (+LR) and the P/N used to predict the evidence of infection |
|-----------------|-----------------|-----------------|-----------------|
| P/N ratio  | DENV-1–4 | WNV | SLEV |
| VLP | SMB | VLP | SMB | VLP | SMB |
| +LR |   |   |   |   |   |   |   |
| < 2 | 6.5 | 4.3 | 2.5 | 1.6 | 2.2 | 1.7 |
| ≥ 2 | 6.2 | 6.5 | 7.5 | 3.3 | 10.0 | 2.4 |
| ≥ 3 |   | 12.5 | 7.5 | 4.4 | 20.0 | 3.4 |
| ≥ 4 |   |   | 15.0 | 6.6 |   | 8.5 |
| ≥ 5 |   |   |   |   |   | 13.1 | 17.0 |
| PPV (%) | ≥ 3 | 100.0 | 92.3 | 83.3 | 63.6 | 71.4 | 41.7 |
| NPV (%) | ≥ 3 | 81.3 | 100.0 | 100.0 | 78.6 | 100.0 | 100.0 |

DENV, Dengue virus; +LR, Positive likelihood ratio; NPV, Negative predictive value; P/N, Positive to negative ratio; PPV, Positive predictive value; SLEV, St Louis encephalitis virus; SMB, Suckling mouse brain antigen; WNV, West Nile virus; VLP, Virus-like particle
The measurement of assay performance by ROC analysis indicated a statistically significant difference between VLPs and SMB antigens used for detection of WNV and the mixture of DENV-1 to -4. VLPs of all seven flaviviruses had lower cut-off P/N ratios than the corresponding SMB antigens (data not shown). The higher PPV and +LR for VLPs also indicated that these antigens performed better than SMB antigens in the MAC-ELISA. Cell lines that continuously secrete VLPs are therefore significantly improved sources of flaviviral serodiagnostic antigens compared with the traditional sources of virus-infected tissue culture or SMB.

The presence of antibodies developed in response to previous flavivirus infection or vaccination complicate flavivirus serodiagnosis. Differentiation between primary and secondary infections is possible by determining the ratio of IgM/IgG in acute-phase sera (Innis et al., 1989; Shu et al., 2003). This requires the simultaneous testing of serum specimens for both IgM and IgG by ELISA. Determination of a fourfold or greater increase in IgM and/or IgG antibody titres between paired sera collected during the acute and convalescent phases is the most reliable method for determination of an active flavivirus infection, but this requires the collection of at least two serum samples from an infected individual, which may not be possible in all cases. A protocol to determine the IgG antibody avidity of a single serum sample has been proposed to differentiate primary and secondary DENV infection (de Souza et al., 2004). This protocol involves treatment of antigen–antibody complexes with a urea solution as the dissociation agent to determine the avidity index of samples. In primary tick-borne encephalitis virus infections, specific IgG antibodies with low avidity are initially produced, with the avidity generally increasing during the maturation of the humoral immune response (Gassmann & Bauer, 1997). If the virus-specific IgG antibody present in a secondary response has higher avidity than that of cross-reactive antibody, it might be possible to reduce the detection of cross-reactive antibodies in ELISA by treatment with urea. However, treatment with urea increases the complexity of the assay without addressing the fundamental problem of cross-reactive antibodies that recognize cross-reactive epitopes on the current serodiagnostic antigens, VLPs as well as virus-infected antigens.

Reduction of cross-reactive epitopes on the VLPs by structure-based mutagenesis (Crill & Chang, 2004) may allow for use of a single assay in DENV serotyping and differentiation between primary and secondary infections. We demonstrate here that some flavivirus VLP antigens are more specific than SMB antigens. Replacement of the virus-infected SMB antigen preparations with VLP antigens in conventional ELISAs may be the first step in reducing the detection of cross-reactive antibodies. This cross-reactivity may be further reduced by identifying and modifying cross-reactive epitopes in these VLPs.

The establishment of a flexible flavivirus expression vector has given us the ability to efficiently produce VLPs for several flaviviruses. By far the greatest asset of this system is the potential for a vaccine application. As described previously for our JEV construct (Chang et al., 2000; Hunt et al., 2001), there is a two-pronged vaccine application. The plasmid construct has been shown to be an effective DNA vaccine, while the VLP antigen has promise as a stand-alone biosynthetic subunit immunogen. Use of VLPs in a DNA prime-VLP antigen boost vaccination regimen may additionally enhance vaccine potency.
References
Davis BS et al. (2001). West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. Journal of Virology, 75:4040–4047.
Shu PY et al. (2003). Comparison of capture immunoglobulin M (IgM) and IgG enzyme-linked immunosorbent assay (ELISA) and nonstructural protein NS1 serotype-specific IgG ELISA for differentiation of primary and secondary dengue virus infections. Clinical and Diagnostic Laboratory Immunology, 10:622–630.
PROBLEMS ENCOUNTERED IN THE MOLECULAR DETECTION OF DENGUE VIRUSES

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Introduction
The importance of the dengue viruses as disease-causing agents for dengue fever and for dengue haemorrhagic fever and dengue shock syndrome has become increasingly recognized. The global prevalence of dengue has grown dramatically in recent decades and the disease is now endemic in more than 100 countries. It is currently estimated that 50–100 million cases of dengue fever occur each year in tropical and subtropical countries, while incidence of the more serious clinical manifestations, dengue haemorrhagic fever and dengue shock syndrome, is also on the increase. Epidemic dengue is now characterized worldwide by hyperendemicity, or the co-occurrence of multiple dengue serotypes in the same locality. Owing to the lack of a vaccine or a cure for dengue fever, it is important to develop effective diagnostic methods and laboratory-based surveillance systems for the early detection of infection and to provide an early warning of dengue fever epidemics. So far, a well-developed molecular biology laboratory method, reverse transcriptase-polymerase chain reaction (RT-PCR) has been widely used for detecting all four serotypes of dengue virus (DENV). The diagnosis of dengue infection by RT-PCR is based on the amplification of specific regions of the DENV genome, followed by electrophoresis in agarose gel and visualization of the products under ultraviolet light after staining DNA with ethidium bromide.

The DENV molecular typing method, RT-PCR, described by Lanciotti (Lanciotti et al., 1992) has been used in our laboratory for more than 10 years. However, we have observed some non-specific results when using this RT-PCR to detect infection. For example, some samples showed evidence for multiple infections by two different dengue serotypes—in most cases, DENV-1 plus another serotype. Additionally, some samples gave positive results for DENV-1 by serological tests, or showed a DNA fragment of the correct size after first-round PCR amplification, but negative results by the nested PCR. These non-specific results thus interfered with our ability to accurately detect infection using this technique, and we determined to resolve the problems encountered.

Since the GenBank database does not contain sufficient sequence information to allow the sequence alignment analyses necessary for the design of new primers to detect Thai DENV variants, it was necessary to establish an adequate sequence database for Thai variants. Recently, we sequenced the envelope gene of 325 DENV isolates (98 DENV-1, 105 DENV-2, 68 DENV-3 and 53 DENV-4) and the complete genome of 32 DENV isolates (10 DENV-1, 10 DENV-2, 6 DENV-3 and 6 DENV-4) taken from Thai patients during 1973–2002 in a study of the molecular epidemiology of DENV circulating in Thailand for the past three decades. Our data established a sequence database of Thai DENV variants, which will serve worldwide as a resource for research on DENV. Based on the information on DENV sequences in our database, we modified Lanciotti’s RT-PCR (Lanciotti et al., 1992) to detect Thai DENV variants. Here we describe some of the modifications performed.

Material and methods

RNA extraction. Virus RNA was extracted from serum using the TRIzol® reagent (Invitrogen, Life Technology), according to the manufacturer’s instructions.

Primers. Lanciotti’s forward primer D1, and reverse primer D2, TS1, TS2, TS3 and TS4 were used. Two new reverse primers, D2n and TS4n were designed based on sequences of Thai DENV in our sequence database. Primer TS1bis, previously described by Reynes et al. (2003), was also used in this study.

RT-PCR. Viral genomic RNA was converted to cDNA using reverse transcriptase from avian myeloblastosis virus (AMV RT, Promega) and a reverse primer D2 or D2n, according to Lanciotti’s RT-PCR method. The first-round PCR products, 511 base pairs (bp) or 656 bp DNA fragments were amplified using AmpliTaq DNA polymerase and primer pairs D1/D2 (Figure 15), and D1/D2n (Figure 17), respectively. These PCR products were amplified from a region that is between the capsid and preM genes.

Nested PCR. The type-specific DNA fragments for DENV-1 (482 bp), DENV-2 (119 bp), DENV-3 (290 bp) and DENV-4 (392 bp, amplified with primer pair D1/TS4, or 389 bp amplified with a primer pair D1/TS4n) were then amplified by AmpliTaq DNA polymerase using the product of the first-round PCR (the 656 bp or the 511 bp DNA fragments) as a template, with mixed primer pairs (one forward primer D1 and four reverse primers: TS1 or TS1bis, TS2, TS3, and TS4 or TS4n) in the nested PCR (the second round of amplification).

Gel electrophoresis. The DNA fragments amplified by the nested PCR were loaded onto a 1.5% agarose gel and subjected to electrophoresis followed by staining of the gel with ethidium bromide. The DNA (including specific DNA bands for each serotype) was visualized under ultraviolet light.

**Figure 14.** Alignment of 15 sequences of DENV-1 isolated in Thailand with the Thai DENV-1 consensus sequence (obtained from our sequence database and GenBank) included as a reference and the sequences of the Lanciotti primer, TS1, and the new primer, TS1bis, for the first-round PCR.

<table>
<thead>
<tr>
<th>Identity</th>
<th>Sequence</th>
<th>Identity</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>D1</td>
<td>D1</td>
<td>D2</td>
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<td>D9</td>
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Identities at nucleotide positions are shown by dots. The sequence numbering was based on the genomic sequences of the consensus of Thai DENV-1. Mutations in the target region are shown in boxes. The first ten DENV-1 samples were isolated from Bangkok, and the last five were isolated from Kamphang Phet Provincial (KPP) hospital. The primer sense is indicated by the horizontal arrow.
Results and discussion

Using a new reverse primer for nested PCR amplification of DENV-1. Some of our DENV-1 isolates gave negative results by Lanciotti’s nested PCR, but positive results by the first-round PCR (Figure 14) and serological tests (data not shown). We noticed that it had been reported that Lanciotti’s RT-PCR did not detect DENV-1 variants in Cambodia, owing to the presence of a point mutation (Reynes et al., 2003). It was probable that we faced the same problem for DENV-1 variants in Thailand. The sequence alignment for these samples (Figure 14) showed that there are some mismatches between the sequences of Thai DENV-1 and the primer TS1. In particular, a point mutation occurred at the 3’ end of primer TS1, which thus has a reduced sensitivity for the detection of some Thai DENV-1 variants in the nested PCR (Figure 15, section B). This point mutation is located at the penultimate base of the 3’ end of the reverse primer TS1 (Figure 14), and thus greatly affects the binding efficiency of DNA polymerase, resulting in unsuccessful amplification and negative results by the nested PCR (Figure 15, section B). To eliminate this mutation, a new reverse primer, TS1bis, designed by Reynes et al. (2003), was used to replace the original one (TS1) in the nested PCR to detect DENV-1 (Figure 14). Nine samples that were known to be positive for DENV-1, isolated from Kamphaeng Phet (KPP) hospital (northern Thailand), were tested using the original primer TS1 and the new primer TS1bis on the same DNA template in the nested PCR (Figure 15, sections B and C). The 482 bp DNA fragment was observed when TS1bis was used (Figure 15, section C), but not when TS1 was used (Figure 15, section B). Our data clearly showed that primer TS1bis was better able to detect Thai DENV-1 variants than was TS1.

FIGURE 15. Agarose gel analysis of the DNA products obtained by RT-PCR and nested PCR from DENV-1 RNA samples isolated in Thailand

(A) After amplification with consensus primers D1 and D2 in the first-round PCR.
(B) After nested PCR amplification with the original (Lanciotti) type-specific primer TS1.
(C) After nested PCR amplification with the new type-specific primer TS1bis.
Lane 1: control mixture of DENV-1–4 (103 PFU/ml). Lanes 2–10: sera from Kamphaeng Phet Provincial hospital (30247/03, 30262/03, 30269/03, 30270/03, 30278/03, 30288/03, 303*B/03, 303*B/03, 303*B/03, 30468/03 respectively). Lane 11: control mixture of DENV-1–4 (103 PFU/ml). Lane 12: negative extraction control. Lane 13: negative RT-PCR control. Lane 14: molecular weight markers (100 bp ladder). Sizes of DNA fragments are given in bp.
**Elimination of a 511 bp interfering DNA band from the first-round PCR amplification.** In the detection of infection with DENV by the Lanciotti RT-PCR (Figure 16), a 511 bp DNA fragment generated in the first-round PCR interfered with the results of the nested PCR (the second-round PCR) for those samples that were positive for DENV-2, DENV-3 and DENV-4 and had a high viral load (Figure 17). The results thus wrongly appeared to show evidence for multi-serotype infections. These ambiguous results were caused by the small difference in size between the 511 bp product of the first round of PCR and the 482 bp DENV-1 product of the second round of PCR. The 29 bp difference between these two DNA fragments could not be distinguished easily by electrophoresis on a 1.5% agarose gel with a short run time.

**Figure 16.** Schematic diagram of Lanciotti’s RT-PCT for detecting infection with DENV

D1 is a forward primer used for both the first-round PCR and the second-round (nested) PCR. D2 is a reverse primer used only in the first-round PCR. TS1, TS2, TS3 and TS4 are reverse primers used in the nested PCR and designed specifically for the detection of each DENV serotype.

**Figure 17.** Agarose gel analysis of the DNA products obtained from 10 DENV RNA samples collected in Thailand, by RT-PCR using the original (Lanciotti) reverse primer D2 followed by nested PCR

Lane 1: molecular weight markers (100 bp DNA ladder). Lanes 2–5: samples positive for DENV-2, 0070/03, 0289/03, 1134/03, and 1210/03 respectively. Lanes 6–8: samples positive for DENV-3, 0072/03, 1393/03, and 1619/03 respectively. Lanes 9–11: samples positive for DENV-4, 1553/03, 1710/03, and 1860/03 respectively. Lane 12: the RT-PCR positive control, comprising a mixture of DENV serotypes 1–4 (103 PFU/ml). Lane 13: the negative control.
We designed a new reverse primer, D2n, to replace the original one, D2, in the first round of PCR. The position of this new reverse primer was shifted 145 bases downstream from that of the original reverse primer D2, resulting in a 656 bp product in the first round of PCR. (Figure 18). The difference in size between the product of the first round of PCR and the DENV-1 product of the nested PCR was 174 bp, which was sufficient to differentiate the two DNA fragments on agarose gel. Our results showed that the two DNA fragments could be clearly separated by using the modified primer D2n in the first-round PCR and interference was eliminated (Figure 19).

**FIGURE 18.** A schematic diagram of the RT-PCR with modified primer D2n for detecting infection with DENV

A new reverse primer, D2n, for the first-round PCR was designed whose position was shifted 145 bases downstream from that of the original primer, D2. Using this primer, a 656 bp DNA fragment was generated in the first-round PCR. The type-specific DNA fragments (482 bp for DENV-1, 392 bp for DENV-4, 290 bp for DENV-3 and 119 bp for DENV-4) for each serotype were produced by the subsequent nested PCR.

**FIGURE 19.** Agarose gel analysis of the DNA products obtained from 10 DENV RNA samples collected in Thailand, by RT-PCR using the new reverse primer D2n followed by nested PCR

Lane 1: molecular weight markers (100 bp DNA ladder). The DENV-positive samples used were as the same as those shown in Figure 17. Lanes 2–5: samples positive for DENV-2, 0070/03, 0289/03, 1134/03, and 1210/03 respectively. Lanes 6–8: samples positive for DENV-3, 0072/03, 1393/03, and 1619/03 respectively. Lanes 9–11: samples positive for DENV-4, 1553/03, 1710/03, and 1860/03 respectively. Lane 12: the RT-PCR positive control, comprising a mixture of DENV serotypes 1–4 (103 PFU/ml). Lane 13: the negative control.
Design of a new reverse primer for detecting DENV-4 in the nested PCR, to avoid priming with Thai DENV-2 variant. Sample D2Th0551/03, isolated from a Thai patient in 2003, gave a positive result for DENV-1, DENV-2 and DENV-4 infection according to Lanciotti’s RT-PCR, while it only gave a positive result for DENV-2 according to the serological test. Did the patient really have a concurrent infection with multiple serotypes or was this just a non-specific result? After sequence alignment analysis, we found that the type-specific primer TS4 binds to the sequence of the D2Th0551/03 at nucleotide positions 497–512 (Figure 20), which might explain the non-specific result for DENV-4. The apparent positive result for DENV-1 was probably caused by the presence of the 511 bp DNA fragment from the first round of PCR. In order to test our hypothesis, we designed a new TS4 primer, TS4n, whose position was shifted two bases upstream (Figure 21). Sample D2Th0551/03 was re-tested using primers D2, D2n, TS2, TS4 or TS4n in both PCRs (Figure 20). Three DNA products of the size expected for DENV-1, DENV-4 and DENV-2 fragments were observed (Figure 22, lane 3) when Lanciotti’s primers were used. However, the 511 bp fragment was eliminated when primer D2 was replaced with D2n (Figure 22, lanes 4 and 5). The non-specific DNA fragment (393 bp) for DENV-4 was removed when primer TS4 was replaced with TS4n (Figure 22, lane 5). These results confirmed our hypothesis.

Our results indicate that, in view of the geographical and temporal variation in DENV sequence, a sequence database for viruses circulating in a particular locality should be established in order to serve as an information resource for the design of specific and sensitive primers or probes for the diagnosis and surveillance of dengue by molecular approaches in the laboratory.

**FIGURE 20.** Alignment of the sequences of one Thai DENV-2 sample (D2Th0551/03; isolated in 2003) with the sequences of primers TS2 and TS4

The mismatched bases between the sequence of D2Th0551/03 and the primer TS4 are shown in boxes. The primer sense is indicated by the horizontal arrow.
**FIGURE 21.** Alignments of the sequences of the original primer TS4 and the new primer TS4n with the sequences of the consensus Thai DENV-4 and six samples positive for DENV-4, and with a sample positive for DENV-2 (D2Th0551/03)

All mismatched bases are shown in boxes. The primer sense is indicated by the horizontal arrow.

**FIGURE 22.** Agarose gel analysis of the DNA products obtained from DENV-2 RNA sample D2Th0551/03 by RT-PCR and nested PCR

Lane 1: molecular weight markers (100 bp DNA ladder). Lane 2: The RT-PCR positive control, comprising a mixture of DENV serotypes 1–4, amplified using Lanciotti’s type-specific mixed primer pairs. Lane 3: the product of the nested PCR for sample D2Th0551/03 using Lanciotti’s type-specific mixed primer pairs. Lane 4: the product of the nested PCR for sample D2Th0551/03 using the new reverse primer D2n in the first-round amplification and the original type-specific primer TS4 in the second-round amplification. Lane 5: the product of the nested PCR for sample D2Th0551/03 using both new primers D2n and TS4n. Lanes 6 and 7: the positive (comprising a mixture of DENV serotypes 1–4) and negative controls, respectively, amplified using both new primers D2n and TS4n.

References


Chips and other new technologies
RAPID DETECTION AND DIFFERENTIATION OF DENGUE VIRUS SEROTYPES BY RT-LAMP

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Introduction
Dengue virus is the most widely prevalent mosquito-borne flavivirus in tropical and subtropical regions of Asia, Africa and Central and South America. There are four distinct serotypes, DENV-1, DENV-2, DENV-3, and DENV-4, producing a spectrum of illness ranging from inapparent infection to moderate febrile illness, to severe and fatal haemorrhagic disease. In recent years, dengue fever and its more serious forms, dengue haemorrhagic fever and dengue shock syndrome, have emerged as a major public health problem owing to their expanding geographic distribution and increased epidemic activity (Gubler, 1997). Isolation of the virus is difficult and methods for detection of antibody have limitations. Therefore, there is a great demand for improved and early detection of dengue virus infection in the acute phase of illness, in order to provide timely clinical treatment and allow etiologic investigation and disease control.

During the past decade, various nucleic acid amplification techniques, such as RT-PCR, nested PCR, Taqman® real-time RT-PCR, SYBR Green real-time RT-PCR and NASBA have been developed to address the need for rapid and accurate identification of dengue virus to serotype level (Morita et al., 1991; Lanciotti et al., 1992; Sudiro et al., 1997; Chan & Fox, 1999; Houng et al., 2000; Shu et al., 2003). Despite the obtainable magnitude of amplification, these PCR-based methods require either high precision instruments for the amplification, or elaborate methods for detection of the amplified products. In addition, these methods are often cumbersome to adapt for routine clinical use, especially in peripheral health-care settings and private clinics. The loop-mediated isothermal amplification (LAMP) assay is a simple diagnostic tool emerging as a powerful gene amplification technique for the rapid identification of microbial diseases (Notomi et al., 2000). The present report describes the development and evaluation of a simple, rapid and cost-effective one-step, real-time and quantitative RT-LAMP assay for the rapid detection and differentiation of dengue virus serotypes. Data on the sensitivity, specificity of the method are reported, and feasibility of the technology for clinical diagnosis of dengue virus infection is discussed.

Materials and methods
Viruses. The virus strains of four dengue virus serotypes (DENV-1, Hawaii; DENV-2, ThNH7/93; DENV-3, PhMH-J1-97; DENV-4, SLMC 318) were used in the present study. The viruses were propagated in Ae. albopictus clone C6/36 cells (Igarashi, 1978) and virus titres were determined by plaque assay in LLCMK2 cells, according to standard protocols.

Serum samples from human patients. The acute-phase serum samples used in this study were collected from patients with confirmed or suspected dengue during epidemics from the Philippines (2000–2003), Bangladesh (2002) and India (2001–2004). In addition, a panel of serum samples collected from healthy volunteer blood donors in the Philippines was also included to act as negative controls.

RNA extraction. Genomic viral RNA was extracted from 140 µl of infected culture
supernatant with a known number of plaque-forming units (PFU) of virus and 50 µl of patient serum samples using the QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer’s protocol. The RNA was eluted from the QIAspin columns in a final volume of 100 µl of elution buffer and was stored at -70°C until used.

**Design of dengue virus serotype-specific RT-LAMP primers.** The serotype-specific oligonucleotide primers used for RT-LAMP amplification of dengue viruses were designed from the 3’-noncoding region. A set of six primers comprising two outer, two inner and two loop primers that recognize eight distinct regions on the target sequence was designed employing the LAMP primer-designing support software program (Net laboratory, Japan, http://venus.netlaboratory.com).

**RT-LAMP.** The RT-LAMP reaction was carried out in a total volume of 25 µl of reaction mixture using the Loopamp RNA amplification kit (Eiken Chemical Co. Ltd., Japan) at 63°C for 60 min in a Loopamp real-time turbidimeter (LA-200, Teramecs, Japan). The analysis of each sample was performed in a set of four tubes each having the primer mix for a particular serotype. Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed. The real-time monitoring of RT-LAMP amplification of dengue virus template was observed via spectrophotometric analysis by recording optical density (OD) at 400 nm at every 6 seconds, with the help of the Loopamp real-time turbidimeter. A sample having time of positivity (Tp) values of ≤30 min and turbidity of above the threshold value of ≥0.1 was considered to be positive. In addition, the amplification was also monitored by agarose gel analysis, as well as by naked-eye inspection by observation of colour change after the addition of 1 µl of SYBR Green I dye to the tube.

**RT-PCR and nested PCR.** In order to compare the sensitivity and specificity of the RT-LAMP assay, one-step serotype-specific RT-PCR was performed on serum samples from confirmed dengue patients, employing the serotype-specific primers of Morita et al. (1991). In the case of samples from patients with suspected dengue, after the initial amplification by RT-PCR with dengue group-specific consensus primers, the second round of amplification was carried out by nested PCR employing serotype-specific internal primers, according to the protocol described by Lanciotti et al. (1992). The amplification was carried out in a total volume of 50 µl of reaction mix with the TaKaRa LA Taq™ PCR kit (TAKARA BIO INC, Japan), and using both Revertra RTAce (Toyobo, Japan) and LA Taq DNA polymerase (Takara, Japan) and 50 pmol of forward and reverse primers with 2 µl of RNA, according to the manufacturer’s protocol.

**Results and discussion**

A set of six primers for each serotype was designed from the 3’-noncoding region, taking into account mismatches among strains, and taking care to avoid primer-dimer formation. The amplification was observed as a ladder-like pattern on the gel owing to the formation of a mixture of stem-loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Figure 23). The Tp as observed through real-time monitoring was found to be 14 min for DENV-1, 16 min for DENV-2, 12 min for DENV-3 and 15 min for DENV-4 (Figure 24). The monitoring of RT-LAMP amplification was also carried out by naked-eye inspection after addition of 1 µl of SYBR Green I dye to the amplified products. The change from the original orange colour of the SYBR Green-I to yellow was indicative of a positive amplification reaction. Enhanced detection was achieved under UV light where the green fluorescence was associated with a positive reaction.
The sensitivity of the RT-LAMP assay was markedly higher than that of the conventional RT-PCR; the RT-LAMP assay was found to be 10–100-fold more sensitive than RT-PCR, with a detection limit of 0.01–10 PFU of virus. The detection limit of the serotype-specific primers were determined to be 10 PFU for DENV-1, 0.01 PFU for DENV-2 and DENV-3 and 0.1 PFU for DENV-4, respectively. None of these primer sets, however, showed any amplification with JEV, WNV and SLEV RNA templates, thereby indicating the specificity for each dengue virus serotype. Further authenticity of the amplified product of each serotype was established by digesting with restriction enzyme (Ban II) having one cutting site at one end of the selected target of all the four serotypes. As depicted in Figure 23, the size of the resulting digested product was in good agreement with the predicted size for each serotype, i.e. 109 bp for DENV-1; 132 bp for DENV-2; 172 bp for DENV-3 and 186 bp for DENV-4.

The applicability of RT-LAMP assay to the detection and differentiation of dengue virus serotypes in patient serum samples was validated by evaluating with acute-phase serum samples from dengue fever and dengue haemorrhagic fever patients infected with all the four serotypes, and the results were compared with those obtained by conventional RT-PCR. The percentage of serum samples that gave a positive result in different test systems was 37%, 33% and 30% by RT-LAMP, RT-PCR and virus isolation, respectively (Table 13). All the confirmed cases of dengue were correctly identified by RT-LAMP, with 100% concordance of results with those of conventional RT-PCR. The sensitivity and specificity of RT-LAMP assay for detecting viral RNA in patient serum samples with reference to virus isolation was 100% and 91%, respectively. It was also observed that none of the four dengue serotype-specific RT-LAMP primer sets showed cross-reaction with healthy serum samples analysed in this study, thereby establishing the specificity of the dengue RT-LAMP assay.

The execution of the RT-LAMP reaction and the measurement of its turbidity are extremely simple compared with the existing real-time Taqman® RT-PCR and NASBA assays that require fluorogenic primers and probes, as well as expensive detection equipment (Notomi et al., 2000; Mori et al., 2001; Nagamine et al., 2002). One of the most attractive features of the RT-LAMP assay is that monitoring of amplification can be accomplished by SYBR Green I dye-mediated naked-eye visualization. Particularly important is the substantial reduction in time required for the confirmation of results; this is less than 1 hour for the RT-LAMP assay compared with 3–4 hours for the RT-PCR assay. In conclusion, the RT-LAMP assay developed in this study allowed the rapid and accurate identification of dengue virus serotypes. As it can be performed simply, without sophisticated equipment, it will be a valuable tool for the rapid detection and differentiation of dengue virus serotypes in well-equipped laboratories and small-scale clinical laboratories, as well as in peripheral health-care settings in developing countries.
**FIGURE 23.** The products of dengue virus serotype-specific RT-LAMP after restriction enzyme digestion and 3% agarose gel electrophoresis

![Image of gel electrophoresis](image)

Lane M, 100-basepair (bp) DNA ladder (Sigma Genosys, Japan); Lane 1, DENV-1 RT-LAMP amplification; Lane 2, Restriction enzyme (RE) digestion of DENV-1 RT-LAMP product (Ban II)—109 bp; Lane 3, DENV-2 RT-LAMP amplification; Lane 4—RE digestion of DENV-2 RT-LAMP product (Ban II)—132 bp; Lane 5, DENV-3 RT-LAMP amplification; Lane 6, RE digestion of DENV-3 RT-LAMP product (Ban II)—172 bp; Lane 7, DENV-4 RT-LAMP amplification; Lane 8, RE digestion of DENV-4 RT-LAMP product (Ban II)—186 bp; Lane 9, Negative control without target RNA.

**FIGURE 24.** Real-time amplification chart for dengue virus serotype-specific RT-LAMP assay, depicting the kinetics of each serotype with regard to time of positivity

![Image of real-time amplification chart](image)

- **DENV-1**
- **DENV-2**
- **DENV-3**
- **DENV-4**
- Negative control
TABLE 13. Comparative evaluation of the results of dengue virus serotype-specific RT-LAMP assay with those of RT-PCR, nested PCR and virus isolation for detection of dengue virus in acute-phase patient serum samples

<table>
<thead>
<tr>
<th>Type of dengue cases</th>
<th>Virus serotype</th>
<th>No. of samples</th>
<th>No. of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT-LAMP</td>
<td>RT-PCR</td>
</tr>
<tr>
<td>Confirmed ^b</td>
<td>DEN-1</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>DEN-2</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>DEN-3</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>DEN-4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Suspected ^c</td>
<td>—</td>
<td>38</td>
<td>6</td>
</tr>
<tr>
<td>Healthy</td>
<td>—</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>—</td>
<td>83</td>
<td>31</td>
</tr>
<tr>
<td>% positive</td>
<td>—</td>
<td>37</td>
<td>33</td>
</tr>
</tbody>
</table>

Concordance, 100%; Sensitivity, 100%; Specificity, 91%

^a Serum samples collected during day 1–7 after onset of symptoms were referred as acute phase samples.

^b All serum samples were selected from patients with confirmed cases of dengue having positive virus isolation, positive RT-PCR and/or positive IgM ELISA with P/N ratio ≥ 2.

^c Samples from clinically suspected patients referred by Municiop Corporation of Delhi, India for screening were referred as suspected samples.

NA, Not attempted

References


A NOVEL INTEGRATED CIRCUIT CHIP-BASED SENSOR FOR THE DIAGNOSIS OF DENGUE

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We are developing a novel integrated circuit (IC) chip-based assay for the detection of protein and nucleic acids. Our first application of this platform was for the diagnosis of infection with dengue virus. This system utilizes micron-scale paramagnetic beads instead of enzymes and an IC sensor for detection, taking advantage of the flexible technology of micro-electromechanical systems (MEMS), as well as the economies of scale in the computer chip industry to enable development of an inexpensive portable diagnostic device. We use a complementary metal oxide semiconductor (CMOS) array of planar magnetic sensors integrated into the surface of the chip; these sensors are capable of detecting small magnetic beads specifically bound to the surface of the chip. The chip surface has a gold overlay to allow liquid interaction, thus providing an interface with biological molecules. For immunological applications, ELISA-based biochemistry is used, wherein antigens or antibodies are adsorbed to the gold surface, followed by blocking with non-specific proteins or polyethylene glycol. The sample is applied to the sensor well, then biotinylated secondary antibodies or antigens and streptavidin-coated magnetic beads are added. Magnetic beads that bind non-specifically are removed using an inexpensive commercial magnet (magnetic force of 350 pN). The presence of bound magnetic beads is detected using an external magnetic field that excites the beads, and individual sensors detect the resulting local magnetic fields. Each of the 1024 sensors is then interrogated to ascertain the presence of a bound bead. This information is transmitted as easily interpretable numeric results to a portable computer; in future, iterations will be recorded into a hand-held personal digital assistant (PDA) or transmitted via a data network.

Our results have shown that antigen (such as human IgG) can be captured and detected using magnetic beads in our prototype system with a limit of detection of 10 pg/ml, a sensitivity that is similar to that of ELISA. In addition, we have measured human IgG antibodies against DEN, with results that are comparable with those of ELISAs. We are currently optimizing the sensitivity and clinical application of our sensor using assays for the detection of anti-DEN IgG and anti-DEN IgM in human sera. For diagnosis of the viraemic stage of dengue, antigen-capture configurations are being developed for the detection of dengue virus NS1 and E proteins. Finally, we are adapting the platform for the detection of nucleic acid using a laddered system of complementary probes. This should enable dengue virus serotypes to be detected and typed early in infection. One advantage of using IC chip technology is that volume manufacturing reduces costs. Since the device is its own sensor, the need for large, expensive detection equipment is obviated. The simplified protocol with less washing relieves the requirement for a laboratory setting. Thus, the combination of immunology or molecular biology and electrical engineering can create a diagnostic device that is low-cost and portable, making it appropriate for point-of-care diagnosis in resource-poor settings. Application of this platform to the detection of dengue virus antigens or RNA makes feasible the early diagnosis of infection.
Strategic planning
CHALLENGES FOR WHO AND THE SCIENTIFIC COMMUNITY

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In view of the geographic expansion in the incidence of dengue fever and dengue haemorrhagic fever, and the increasing numbers of cases and epidemics, there is an urgent need for useful methods of dengue diagnosis. Diagnosis needs for dengue surveillance, clinical diagnosis and prognosis, studies of pathogenesis and vaccine testing must be carefully determined in order to define research strategies. The identification of sources of financial support and of institutions with the capacity to carry out this research, the development of easy and inexpensive tests and the rapid introduction of these tests into daily practice in countries where dengue infection is endemic are part of this process.

In the current situation, other important aspects also deserve to be mentioned:
• the availability of reference and no-reference reagents
• the evaluation of commercial diagnostic kits and PCR protocols
• the extension and recommendation of standardized protocols for dengue diagnosis
• improving the quality and quantity of proficiency tests
• human capacity building
• the role of the WHO Collaborator Centres
• information exchange between countries, regions and Collaborator Centres
• the availability of serum and virus strain banks
• the extension of advanced technology to WHO Collaborator Centres
• the extension of biotechnological techniques for new and improved diagnostic tools.

The establishment of a network for an integrated approach (standardization and sharing of reagents and protocols), the South–South collaboration (for the development of new technology for dengue diagnosis) and capacity building are important aspects of these aims (WHO, 2002).

Reference
WHO (2002) Biotechnology and Genomics for Improvement of Health in Developing Countries, Havana
DEVELOPMENT OF AND QUALITY ASSURANCE FOR DENGUE POINT-OF-CARE TESTS (POCTS)

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Summary
Point-of-care testing or near-patient testing is the performance of laboratory testing at or near the patient’s bedside. Clinical staff use small and easily-operated medical devices to immediately obtain results that can be used in the decision-making process (Harvey, 1999; Kane, 1999; Kost et al., 1999; Castro et al., 1995). One of the most rapidly growing areas within the medical device industry, POCTs are particularly important for use in the field, resource-poor settings and in cases where short turnaround times are critical. Faulty POCT devices and inappropriate use of these devices present risks to patients and health-care workers through the generation of incorrect results and wrong decisions. It has been established that the majority of rapid tests for dengue are produced without proper standards. Today, however, several initiatives from professional bodies, regulatory authorities and governments aim to improve the quality of all medical devices. The rapid changes that have been realized in the development and evolution of POCTs based on traditional and new technologies provide additional challenges. This presentation highlights the essential elements of a quality control approach to a POCT for dengue, with a focus on lateral flow technology.

Quality control
Quality assurance guidelines for POCTs are available from the European Union Directive, the Food and Drug Administration (FDA), the Centers for Medical Services (CMS), the College of American Pathologists (CAP) and the Joint Committee on Accreditation of Healthcare Organizations (JCAHO).

Manufacturing. The critical elements of a quality-control system are:
• Analytical methods;
• Equipment operation, maintenance and calibration;
• Raw material acceptance procedures;
• Pre-production planning procedures;
• Pilot preparation procedures;
• In-process and final product testing procedures (must include potentially cross-reactive and interfering substances);
• Storage and control of in-process and finished products;
• Product release and shipping procedures.

Figure 25 depicts a lateral flow test strip construct. The physical components requiring quality control are the sample medium, conjugate medium, chromatographic medium, desiccants, moisture barrier, cassettes, if used, and equipment. In-process materials include antigens, antibodies, nano-particles or colloids, buffers, critical chemicals for production and blocking agents. In order to perform quality control, material specifications and acceptance criteria must be established first!

General stability
Quality control must be conducted in the context of known product stability. Real-time and accelerated stability must be conducted at various temperatures.

Accelerated stability. This is required for prediction of shelf-life and the possible
adverse effects of shipping. The rationale is to exacerbate the conditions that might potentially harm the device. Examples of stress conditions are heat, humidity and light.

**Accelerated stability predictions.** These predictions can be made from theory, chemical kinetics and cumulative experience. The most common method used to predict stability is the Arrhenius equation, a method that relates the constant to the activation energy and temperature. This is given by the equation:

\[ k = Ae^{-\frac{Ea}{RT}} \]

where

- \( Ea \) = activation energy
- \( T \) = temperature (Kelvin)
- \( R \) = gas constant (8.314 J K\(^{-1}\) mol\(^{-1}\))
- \( A \) = frequency factor (L mol\(^{-1}\) s\(^{-1}\))

**Regional centre and end-user requirements**

**Quality control guidelines.** Quality control must be conducted in larger centralized labs, in the doctor’s office or by the field user. Test performance must be checked at the laboratory upon receipt and at intervals within the recommended shelf-life. It is important to monitor transport and storage temperatures at each location. Personnel using the tests should be trained and periodically monitored regarding procedure and interpretation of results.

**Controls for quality control.** Appropriate external controls must be used for quality control testing. The controls recommended are:

- Commercial or in-house prepared controls;
- Proficiency testing specimens with confirmed results;
- Controlled strains of microorganisms;
- Calibrators of lot numbers or concentrations that are different to those used to calibrate the test system;
- Patient specimens with established values.

There should be agreement on the reference standard to be used to measure performance, and minimum acceptable sensitivity and specificity standards must be established. For testing, it is suggested that a combination of a dengue sero-
conversion panel, a dilution series of a positive sample, and a borderline panel be used. In addition, access to a dengue strain repository and a Flavivirus panel would be an asset.

**Sensitivity and specificity.** It is important to use the recommended type and number of samples for the kit under evaluation, and to review PPV data from the manufacturer or other publications, including:
- PPV: proportion of reported cases that have the health-related event under study
- Low PPV (frequent ‘false positive’ results in case reports) in dengue will cause difficulty in the test interpretation.

**Precision.** Examples for intra-assay and inter-assay protocols are suggested below:
- Intra-assay: at least three different serum, plasma or whole blood samples represented by a negative, borderline and high positive result; replicates = 8–10;
- Inter-assay: at least three different serum, plasma or whole blood samples represented by a negative, borderline and high positive result; replicates = 4–6.

No discrepancies should be obtained under these testing conditions.

**Conclusion**
The totality of features and characteristics that embody rapid medical devices for use in vitro must be carefully validated by manufacturers and end-users. In order to sustain reliable and safe, high performance devices, a comprehensive quality control programme must be followed in accordance with established guidelines.

The primary onus is on manufacturers of medical devices, who must establish a solid base of knowledge concerning the technical aspects of the test device and its components in order to consistently produce a reliable product. Furthermore, the ability to troubleshoot appropriately and to solve problems quickly when unexpected deviations are presented must not be overlooked. Equally important is to ensure that the personnel responsible for testing are properly trained to monitor the integrity of the devices and to execute proper good laboratory practice throughout the evaluation process.

Because medical devices for use in vitro are prone to variation during manufacture, shipping and storage, it is our collective responsibility as health-care professionals to ensure that they are frequently monitored for quality at all levels.

**References**
THE ROLE OF WHO/TDR IN FACILITATING THE DEVELOPMENT, EVALUATION AND APPLICATION OF DIAGNOSTICS FOR DENGUE

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The UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) was established in 1975 to conduct research into the development of new tools and strategies for the control of tropical diseases, and to provide training for researchers from countries where such diseases are endemic. The tools, which include drugs, vaccines and diagnostics, are developed through public–private partnerships. The mission of the Diagnostics Research and Development (DRD) unit in TDR is to facilitate the development, evaluation and application of tropical disease diagnostics that are appropriate for use in developing countries.

The pathway from research and development of a new diagnostic tool to its use in patients involves multiple stages, from target discovery and reagent research, through product development and evaluation, regulatory approval, demonstration of utility and impact, to its sustainable adoption for case management and disease control in countries where the disease is endemic (Figure 26, left column). For diseases with profitable markets in developed countries, this pathway is largely driven by biotechnology companies. Motivated by the expectation of return for investments, companies seek out the scientific, regulatory, and market information they need, develop the product, conduct the necessary trials for regulatory approval, and find public- and private-sector partners to conduct operations and impact studies to encourage the uptake of new products. These companies direct and coordinate the ‘research and development to patient use’ process for their products, drawing in partners as necessary to achieve their goal of getting an effective product into the hands of people who need and can afford to pay for it.

Unfortunately, for diseases that are predominantly endemic in developing countries, such market-driven incentives rarely exist. Companies with interests in these areas tend to be small and under-resourced. They are often not familiar with the specific diagnostic product specifications that would address public health needs, have limited or no access to reagents, strains and specimens to accelerate product research and development, and lack partners in disease-endemic countries to conduct the necessary clinical trials for regulatory approval and for its sustainable adoption. (Figure 26, Barriers). The result is that efforts towards diagnostics research and development for diseases prevalent in developing countries often lack coordination, with duplication and gaps interrupting the ‘research and development to patient use’ pathway.

Public-sector interests and investments are then needed to overcome these barriers to the development of diagnostics appropriate for developing countries. Most public-sector research programmes tend to be investigator-driven, with relatively little public grant money going explicitly into diagnostic product development. Public–private partnerships are needed to take on the responsibility of advocating for funding, leveraging the expertise in the private sector with public-sector investments, and providing leadership to coordinate and drive the diagnostics ‘research and development to patient use pathway’ (Figure 26, Possible solutions).

8 www.who.int/TDR
Building on the success of TDR’s effective partnerships with the private sector for drug development, the TDR DRD unit has developed a strategy for working with public and private sectors in facilitating the development, evaluation and application of diagnostics for developing countries. As shown in Figure 27, DRD seeks to facilitate:

Test development through defining needs and converting science and technology innovation into much needed pro-poor products

- Defining needs: DRD convenes expert consultations to define diagnostic needs and product characteristics that would meet public health needs.
- Facilitating research and development: through open competitions for ‘Bright Ideas’ projects, TDR provides seed funding for:
  - Novel diagnostic target or biomarker discovery;
  - Adapting existing tests and technologies to diagnostic formats appropriate for settings in developing countries;
  - Reagent research.
- Facilitate test development: banks of well-characterized specimens and strains from diverse geographic areas are an important means of facilitating test development efforts in both the private and public sector.

**Figure 26.** Barriers and possible solutions to the development, evaluation and application of diagnostics for use in developing countries
TDR does not demand ownership of any intellectual property arising from such funded research, but does stipulate that such tests be made available to developing countries at negotiated prices.

**Test evaluation in populations of intended use around the world to provide evidence for utility and effectiveness**

TDR DRD has established networks of laboratory and field sites for the evaluation of its priority diseases. The quality of these TDR sites is assured through provision of workshops in good laboratory practice and good clinical practice, and of proficiency panels to ensure quality reference standard tests are performed at each evaluation site. All trials are monitored. As field trials are expensive and time consuming, diagnostics are often evaluated first using well-characterized samples from a central specimen bank or from different laboratory sites. Promising tests to be performed by health workers without laboratory training and to determine utility, patient and provider acceptability are then selected for further evaluation in field settings.

**Test introduction and sustainable adoption**

Uptake and sustainable adoption of tools into control programmes depend on multiple factors, of which cost and policy are critical. DRD facilitates test introduction and adoption by:

- Using mathematical models and validation studies to provide evidence for impact and cost–effectiveness of different strategies for test introduction.

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**FIGURE 27. WHO reference laboratory network and strategic issues**
• Working with governments to synthesize and translate the evidence into policy for the sustainable adoption of rapid testing into control programmes.
• Advocating for regulatory harmonization to minimize the number of trials required for regulatory approval in every disease-endemic country so that the cost savings can be translated into lower prices.
• Using the WHO Bulk Procurement Scheme to ensure access for all United Nations Member States and United Nations agencies at affordable prices.

The ultimate goal of this strategy is to facilitate the development and evaluation of diagnostics that meet the public health needs in developing countries and ensure its sustainable adoption. This critical path has already been used for the evaluation and adoption of promising rapid tests for syphilis, six of which have now been included in the WHO Bulk Procurement Scheme, with a 50% reduction in prices. The wider access to quality-assured screening tests has stimulated the initiation of plans to eliminate congenital syphilis; this will make a significant contribution towards achieving the Millennium Development Goals. From the presentations at this workshop, it is clear that there is an urgent need for appropriate diagnostic tools for dengue patient management (early detection and risk assessment), surveillance and vaccine trials. A number of rapid, simple tests for dengue are commercially available in developing countries and there may be opportunities to use these current technologies to make an impact, however, there is limited data on whether these tests are effective. There is a range of laboratory-based diagnostic techniques currently in use, but the lack of standardization of these techniques between different laboratories is a major concern. The consensus of participants at this workshop was that a strategy for dengue diagnostic development and evaluation and a partnership for advocating, developing and implementing the strategy are needed to remedy this situation.

This strategy consists of the following elements:
• Facilitation of test development efforts in both the public and private sector through the establishment of repositories for reagents, dengue strains and specimens to accelerate the test development and evaluation process.
• Evaluation of existing tests (and new tests currently in development) using well-characterized specimens from the repositories above and establishing a network of laboratory and field-evaluation sites whose activities are coordinated and quality-assured by one or two reference laboratories.
• Development of a quality assurance/proficiency scheme for dengue diagnostics to ensure not only the quality of commercial tests sold, but also their processing. This will improve the quality of diagnostic results used for patient management, surveillance and vaccine trials.
• Development of an information network for dengue diagnostics to disseminate up-to-date information on dengue diagnostics and diagnostics development. This can be developed with existing networks for dengue surveillance.

The ultimate goal of this strategy is to develop and make available simple, rapid, quality-assured tests that can be used with clinical data for early case detection and risk assessment without the need for a follow-up visit. Such tests may also be useful for surveillance, but surveillance data can often be obtained from laboratory-based tests. If the diagnostic evaluation scheme is sufficiently rigorous, it is hoped that

9 http://www.who.int/diagnostics_laboratory/procurement/en/
10 http://www.un.org/millenniumgoals/
promising tests can be included in the WHO Bulk Procurement Scheme at negotiated prices. This will ensure wider access as the tests become more affordable.

Table 14 contains a list of proposed activities under each element of the strategy and some proposed timelines for each activity. This represents the first draft of an important initial step for the WHO/TDR and PDVI partnership. The next step is to convene a meeting with industry to obtain input and necessary modifications to the strategy and plan of action.

### TABLE 14. Dengue diagnostics: proposed plan of activities

<table>
<thead>
<tr>
<th>Activities</th>
<th>Time-line in quarters of a year</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Objective 1: Facilitate test development and evaluation</strong></td>
<td></td>
</tr>
<tr>
<td>Activity 1: Refine product specifications and intended use</td>
<td>1</td>
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<tr>
<td>Activity 2: Meeting with industry</td>
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<tr>
<td>Activity 3: Develop a network of specimen/strain repositories</td>
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<tr>
<td>– establish reference laboratories</td>
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<tr>
<td>– establish network of collection sites by open competition</td>
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<tr>
<td>– develop terms of reference for sites/repositories</td>
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<tr>
<td>– initiate collections</td>
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<tr>
<td><strong>Objective 2: To evaluate the performance and utility of existing dengue diagnostics</strong></td>
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<tr>
<td>Activity 1: Development of evaluation scheme</td>
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<tr>
<td>– development of an evaluation protocol</td>
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<tr>
<td>– invite company participation</td>
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<tr>
<td>– establish a network of evaluation sites</td>
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<tr>
<td>– initiate evaluation</td>
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<tr>
<td><strong>Objective 3: To develop a quality assurance/proficiency scheme for dengue diagnostics</strong></td>
<td></td>
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<tr>
<td>Activity 1: Work with reference labs to develop terms of reference for scheme</td>
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<tr>
<td>Activity 2: Define quality assurance/proficiency panels</td>
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<tr>
<td>Activity 3: Initiate scheme</td>
<td></td>
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<tr>
<td><strong>Objective 4: To establish an information network on diagnostics</strong></td>
<td></td>
</tr>
<tr>
<td>Activity 1: Develop collaborative relationships with other interested partners for best means of information dissemination</td>
<td></td>
</tr>
<tr>
<td>Activity 2: Develop a web site for dengue diagnostics</td>
<td></td>
</tr>
</tbody>
</table>
Appendix: List of participants

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