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REVIEW

# Dengue diagnosis, advances and challenges

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**Summary** Dengue diagnosis was one of the topics discussed at the symposium 'The Global Threat of Dengue — Desperately Seeking Solutions' organized during the 10th International Congress of Infectious Diseases held in Singapore in 2002. In this paper, a review is presented focusing on the main advances, problems and challenges of dengue diagnosis.

IgM capture ELISA, virus isolation in mosquito cell lines and live mosquitoes, dengue specific monoclonal antibodies and PCR have all represented major advances in dengue diagnosis. However, an appropriate rapid, early and accessible diagnostic method useful both for epidemiological surveillance and clinical diagnosis is still needed. Also, tools that suggest a prognosis allowing for better management are also needed. Finally, laboratory infrastructure, technical expertise and research capacity must be improved in endemic countries in order to positively influence dengue surveillance, clinical case management and the development of new approaches to dengue control.


The very title of the symposium 'The Global Threat of Dengue-Desperately Seeking Solutions' organized during the 10th International Congress of Infectious Diseases in Singapore in 2002, highlights the devastating impact of dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) as well our current inability to control and prevent this disease.

Today, DF and DHF/DSS are considered the most important arthropod-borne viral diseases in terms of morbidity and mortality. More than 2.5 billion people are at risk of infection and more than 100 countries have endemic dengue transmission. DHF has been reported in 60 of them.[1,2]

The burden of DF and DHF disease is not very well documented; however in 1998 alone, more than 1.2 million cases were reported to the World Health Organization, with south-east Asia, the western Pacific and more recently the Americas being the most affected regions.[2,3]

The emergence and re-emergence of dengue can be attributed to a number of underlying causes. These include demographic and societal changes such as population growth and unplanned urbanization; this can result in large, crowded human populations living in urban centers with substandard housing and inadequate water, sewage and waste management systems. When these factors are combined with increased movement of individuals from endemic areas, the deterioration of effective mosquito control measures, and the limited financial and human resources dedicated to

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the public health infrastructure, dengue can gain a foothold within the population.[4–6]

Dengue virus infections may be asymptomatic, or may lead to undifferentiated fever, DF or DHF/DSS. The incubation period for dengue is four to six days. Infants and young children usually develop an undifferentiated febrile disease that can be accompanied by a maculopapular rash. Older children and adults may develop either a mild febrile syndrome or the classical dengue fever, characterized by fever, headache, myalgias, arthralgia and rash. However, three or four days after the onset of fever, and generally when the fever falls, some patients present bleeding manifestations (at least a positive tourniquet test), thrombocytopenia and hemoconcentration. Hepatomegaly can also be observed. Patients usually recover after fluid and electrolyte therapy. In severe cases, shock is observed, characterized by signs of circulatory failure (weak and rapid pulse, hypotension or narrowing of the pulse pressure, cold and clammy skin and restlessness). Shock is followed by death in 5–10% of cases if rehydration is insufficient or delayed. Plasma leakage is the main characteristic of DHF/DSS.[7–10]

Both syndromes, DF and DHF/DSS, are caused by any of the four dengue serotypes that belong to the family *Flaviviridae*. Dengue viruses are spherical, lipid-enveloped viruses that contain a positive strand RNA genome of approximately 10,200 nucleotides coding for three structural proteins (capsid, membrane and envelope) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5). The envelope protein (E) plays a key role in several important processes including receptor binding, blood cell hemagglutination, induction of a protective immune response, membrane fusion and virion assembly.[11,12]

Today, secondary infection by a different dengue serotype is considered the most significant individual risk factor for DHF/DSS.[13–15] The presence of circulating non-neutralizing, cross-reactive antibodies in a previously immune individual allows for enhancement of infection, favoring the increased entrance of the virus into the target cell through the cell Fc receptor.[16] It seems that T cell responses also play an important role in the pathogenesis of the severe disease. Both CD4+ and CD8+ T cells have been detected in naturally immunized people and cytotoxic dengue-specific CD4+ T cell clones have been shown to have both serotype-specific and serotype-cross-reactive specificities. In this model, previous infection with one dengue serotype results in the presence in the host of cross-reactive antibodies and memory T cells. Once infected by a different serotype, virus-antibody complexes lead to complement activation and enhanced infection of monocytic cells. The targeting of dengue virus-infected monocytic cells by T cells results in the release of cytokines, lysis of cells, and the release of intracellular enzymes and activators, leading to subsequent plasma leakage and shock.[17,18]

Recently, Mongkolsapaya et al., have reported the presence of many dengue-specific T cells of low affinity for the infecting virus and higher affinity for the others. They suggest that a profound T cell activation and death may contribute to the systemic disturbances observed during DHF. They also propose that original antigenic sin in the T cell responses may suppress or delay viral elimination.[19]

The pathogenesis of DHF/DSS is not very well understood nor are the host conditions that favor the severe disease; however, children, females, individuals with chronic diseases such as asthma and diabetes, and whites appear to be at greater risk. Finally, recent reports argue the risk of DHF/DSS is higher if the interval is longer between primary and secondary dengue infection.[20–23]

Among the many topics covered in the symposium were new developments and challenges in the area of dengue diagnosis.

## Applications and implications of dengue diagnosis

Accurate and efficient diagnosis of dengue is important for clinical care, surveillance support, pathogenesis studies, and vaccine research. Diagnosis is also important for case confirmation (DF or DHF/DSS), to differentiate dengue from other diseases such as leptospirosis, rubella, and other flavivirus infections, and for the clinical management and evaluation of patients with severe disease.[24,25]

In conjunction with clinical and epidemiological surveillance, the early detection of dengue circulation or an increase in dengue activity provides to health authorities useful information on time, location, virus serotype and disease severity.[7] The use of good dengue diagnostic tools is critical for laboratory confirmation of DHF/DSS, including the number of case fatalities, determining which strains are involved, and to derive estimates of total incidence following epidemics.[7,26–31]

Finally, dengue diagnosis is of major importance for research into host, virus and vector characteristics, for determining the epidemiological conditions influencing the pathogenesis of the disease, and for vaccine evaluation (phase 1–3 studies).[32–36]

## Current protocols for the diagnosis of dengue infections

Dengue diagnosis can be performed through virus isolation, genome and antigen detection and serological studies. Serology is currently the most widely applied in routine diagnosis. Of course, clinical, geographical, and epidemiological data associated with the patient remain critical considerations when evaluating a laboratory result.

### Serological diagnosis

Dengue infection in a non-previously immune host produces a primary response of antibodies characterized by a slow and low titer antibody response. IgM antibody is the first immunoglobulin isotype to appear. Anti-dengue IgG appears in a low titer at the end of the first week of disease onset, and increases slowly. By contrast, during a secondary infection (dengue infection in a previously dengue or flavivirus immune host), antibody titers rise extremely rapidly and antibody reacts broadly with many flaviviruses.[37] High levels of IgG are detectable even in the acute phase and they rise dramatically over the following two weeks. The kinetics of the IgM response are more varied, appearing late during the febrile phase of illness, often preceded by IgG. Some anti-dengue IgM false negative reactions are observed in secondary infections. According to Pan American Health Organization (PAHO) guidelines,[7] by day five of illness, 80% of cases have detectable IgM antibody, and by day six to ten, 93—99% of cases have detectable IgM that may persist for over 90 days.

Anti-dengue IgM detection using enzyme-linked immunosorbent assay (ELISA) represents one of the most important advances and has become an invaluable tool for routine dengue diagnosis. Specifically, MAC-ELISA (IgM antibody capture ELISA) diagnosis is based on detecting dengue-specific IgM antibodies in the test serum by capturing them using anti-human IgM antibody previously bound on a solid phase.[37—39] In general, 10% false negative and 1.7% false positive reactions have been observed.

Different formats such as capture ELISA, capture ultramicroELISA, dot-ELISA, AuBioDOT IgM capture and dipstick have been developed.[37,40—43] Serum, blood on filter paper,[7,44,45] and more recently saliva are useful for IgM detection if samples are taken within the appropriate time frame (after five days of onset of fever). Different commercial kits[46—53] for anti-dengue IgM and IgG detection are available, with variable figures of sensitivity and specificity (see Table 1).

In a suspected case of dengue, the presence of anti-dengue IgM antibody suggests recent infection. IgM detection is not useful for dengue serotype determination due to the cross-reactivity of the antibody observed even during primary infection. In a series of serum samples of dengue patients from Nicaragua, Panama and Costa Rica, a serotype-specific IgM response was observed in only 15% and 16% of DF and DHF cases respectively, and in 17% and 14% of the primary and secondary cases (Guzman MG, unpublished data). Dengue IgM antibodies also cross-react to some extent with other flaviviruses such as Japanese encephalitis and St. Louis encephalitis and yellow fever.[54,55]

In an attempt to quantify IgM antibodies to arboviruses of medical importance from three virus families (*Togaviridae*, *Flaviviridae* and *Bunyaviridae*), some investigators have used a standardized combined MAC-ELISA using prototype viruses, well-characterized human sera, and broadly group-reactive monoclonal antibody conjugates. This system has resulted in a good approach for rapid screening of human serum samples for various arboviruses.[56]

Clinically, diagnostic seroconversion is defined as a fourfold rise (or fall) in antibodies in paired sera by hemagglutination inhibition (HI), complement

**Table 1** Several commercial kits available on the market for detecting anti-dengue antibodies.

| Commercial kits | Immunoglobulin isotype detection | Format | References |
|---|---|---|---|
| PanBio Dengue Duo | IgM/IgG | ELISA | 48,50,52 |
| PanBio Dengue rapid test | IgM/IgG | Immunochromatographic test | 46,49,51,52 |
| MRL Diagnostic Dengue | IgM | ELISA | 49 |
| Blot IgM™, Diagnostic Biotechnology Ltd. | IgM | Immunoblot kit | 47 |
| Venture Technologies Dengue IgM and IgG Dot Blot kits | IgM/IgG | Immunoblot kit | 50 |
| Integrated Diagnostics | IgM | Dipstick | 51 |
| Dengue Duo Rapid Strip Test, PanBio | IgM/IgG | Immunochromatographic test | 53 |
| UMELISA Dengue IgM | IgM | Ultramicro-ELISA | 41 |

fixation (CF), plaque reduction neutralization technique (PRNT) or ELISA.[7,24,25,57,58] Due to the presence of cross-reactive antigens shared by flaviviruses, specific diagnosis is not possible in most cases. When a serological specific diagnosis is required, PRNT is used, as this assay is the most specific serological tool for the determination of dengue antibodies.[59]

In order to determine the presence and quantity of dengue-neutralizing antibodies, several protocols have been developed; Vero and BHK21 cell lines and carboxymethyl cellulose (CMC) and agarose are frequently used, while some investigators use peroxidase-antiperoxidase (PAP) staining.[60–64] Currently, few laboratories use PRNT in their studies.

In order to implement PRNT, Shu et al. in 2002 standardized an NS1 serotype-specific indirect ELISA to differentiate primary and secondary dengue virus infections and obtained a good correlation between anti-NS1 serotype-specific IgG (determined by ELISA) and PRNT results.[65,66] In contrast, Cardosa et al., 2002, demonstrated that the IgG response against premembrane protein was specific to flavivirus. No cross-reaction was observed when sera were tested from individuals infected with dengue virus or Japanese encephalitis virus. These authors recommended the use of premembrane protein for seroepidemiological studies.[67]

HI is the accepted serological technique[68] however, as it is time consuming, ELISA has become the most frequently used technique for serological studies.

ELISA for anti-dengue IgG detection is currently widely used for classifying cases based on the kind of infection, primary or secondary. Some protocols use serum dilutions to titer anti-dengue IgG. In others, a ratio of IgM/IgG higher than 1.78 is considered a marker of primary infection, and less is considered a marker of secondary infection.[38,69–73]

Relatively recently, some investigators have demonstrated the usefulness of anti-dengue IgA detection as an indicator of recent infection. Talarmin et al. determined the presence of anti-dengue IgM and IgA antibodies in 178 sera from patients with DF.[74] Figures of 100% sensitivity and specificity were obtained. IgA antibodies were detected at day six following the onset of fever until day 25. On average, IgM antibodies were detected at day 3.8 and IgA at day 4.6. On the other hand, Groen et al.[75] also suggest the diagnostic value of IgA serum detection using an immunofluorescence assay (IFA); however, a higher percentage of IgA detection was observed in acute serum samples from secondary cases (62%) when compared to primary cases (17%). Similar results were obtained by Balmaseda et al. when they applied an ELISA to the detection of anti-dengue IgA in sera.[76] On the other hand, Koraka et al. observed significantly higher levels of dengue virus-specific IgE in sera of DHF/DSS, than in DF cases and non-dengue patients. The measurement of these antibodies is proposed as a prognostic marker.[77]

## Virus detection

Dengue viremia is short, is usually observed two to three days before the onset of fever and lasts four to five days later. Therefore, samples for virus isolation must be taken in the first four to five days of the disease.

Serum is the sample of choice for routine diagnosis, however dengue virus can also be detected in plasma, leukocytes and in tissues obtained at autopsy such as liver, spleen, lymph nodes, lung and thymus.[25,78–81] Because dengue virus is heat-labile, appropriate handling of the specimens and prompt delivery to the laboratory is required for successful virus isolation. For short-term storage, specimens may be kept at 4 °C, however for longer storage low temperatures are recommended (−70 °C).

Mosquito inoculation is the most sensitive system for dengue virus isolation and both adult and larval mosquitoes can be used. Generally, *Toxorhynchites* mosquitoes are preferable because of their large size and because they are not haematophagous. Adult male *Aedes aegypti* and *Aedes albopictus* mosquitoes are also useful for virus isolation.[82–86]

Mosquito inoculation for dengue detection is also useful in the quality control of vaccines. Jirakanjanakit et al. inoculated *Toxorhynchites splendens* with a tetravalent live attenuated dengue vaccine and demonstrated that no interference between serotypes occurred in infected mosquitoes.[87]

Because of the technical skill and special containment required for direct mosquito inoculation, cell culture is preferable for routine diagnosis, despite the greater sensitivity of methods employing mosquitoes. It was clear from the first reports of their use that mosquito cell cultures were ideal for dengue virus isolation. Different cell lines and cell clones have been studied, however a cell line cultured from *Aedes albopictus* (C6/36) has become the host cell of choice for routine dengue virus isolation, although the *Aedes pseudoscutellaris* cell line AP61 has also been successfully used.[88–96]

Rodriguez et al.[97] used a rapid centrifugation technique to isolate the dengue virus cultured in C6/36 cells and obtained 16.6% more isolates than with the conventional method. Of even more importance, this method was useful for isolating the virus from tissue samples derived from fatal cases

of dengue.[79] These authors reported the recovery of 42.8% of viral isolations from these tissue samples.

Mammalian cell cultures such as Vero cells, LLCMK2 cells and others have also been employed with less efficiency.[24,25,98]

The oldest and least sensitive method for isolating the virus is through the intracerebral inoculation of suckling mice—this is only used when no other methods are available. Although many animals develop symptoms or signs indicating encephalitis, a large number of animals exhibit no signs of illness.[99,100] In terms of virus isolation, the use of mosquito cell lines represents the most important contribution to dengue diagnosis.

Virus identification is generally accomplished using immunofluorescence techniques with serotype-specific monoclonal anti-dengue antibodies on mosquito head squash, infected cells or brain tissues from mice. Specific monoclonal antibodies available at the American Type Culture Collection and at World Health Organization Collaborating Centers have simplified the identification of these viruses. In general, samples are first tested by IFA using a polyclonal antibody and those positives are then re-tested with the four serotype-specific monoclonal antibodies.[101–104] Some strains are not easily identified because of low virus concentration, and so some investigators have recommended one or two passages through a cell culture system in order to increase the viral concentration.[104]

Flow cytometry has recently been reported as a useful method for dengue 1 identification. It allows the virus to be identified ten hours earlier than an IFA where anti-NS1 monoclonal antibodies are used.[105]

## Antigen detection

IFA and radioimmunoassay (RIA) have detected dengue viral antigens, however, the low sensitivity of these tests has not allowed their application in routine diagnostic purposes.[80,106]

In recent years, some sensitive systems have been standardized in a typical ELISA format. In 1995, Malergue and Chungue applied a streptavidin-biotin amplified fluorogenic ELISA to the detection and identification of the dengue 3 antigen in serum. This ELISA showed a sensitivity of 90% and specificity of 98% when compared to virus isolation.[107] Later, Kittigul et al. demonstrated that the dengue antigen could be detected at a higher frequency in peripheral blood mononuclear cells (PBMC) compared to sera (53.8% as opposed to 18.9%). These investigators also made use of a biotin-streptavidin ELISA.[108] More recently, attention has been focused on NS1 antigen detection. Young and co-workers standardized a capture NS1 ELISA and demonstrated the presence of high levels of NS1 in the acute phase serum of patients suffering from secondary infection. They suggested that NS1 antigen detection could be useful for early diagnosis and also as a marker of viremia.[109] Similar results were obtained by Alcon et al.[110] Finally, Libraty et al., demonstrated the effectiveness of NS1 detection as a predictor of DHF. NS1 levels in plasma correlated with viremia levels and were higher in DHF patients than in those with DF.[111]

A commercial kit based on two ELISAs for antigen detection (blue kit) and identification (red kit) has also been recently produced. According to the manufacturer, the sensitivity and specificity is 84% and 89% for the blue kit and 91% and 93% for the red kit (Globio Blue and Red Kit for antigen detection, Globio Corp., Beverly, MA, USA).

Immunohistochemical techniques (using horseradish peroxidase or alkaline phosphatase labels) have been shown to be useful for dengue antigen detection in formalin-fixed paraffin-embedded tissue samples, although this technology is not widely used for diagnosis in dengue endemic countries.[112–114]

## Genome detection

In recent years, PCR (polymerase chain reaction) has become an important tool for the diagnosis of dengue, for laboratory screening including entomological surveillance, and for molecular epidemiological studies.[115] It has also proven useful as a research tool in pathogenesis, antiviral drug and vaccine studies.

DNA amplification is preceded by a reverse transcription reaction, producing cDNA from the target RNA. Dengue RNA has been detected by PCR in serum, plasma, infected cells, infected mosquito larvae, mosquito pools, fresh and paraffin-embedded tissues and formalin-fixed tissue.[29,30,115–117]

Several PCR protocols have been developed. Many of them apply a combination of four serotype-specific oligonucleotide primer pairs in a single reaction tube or use a universal dengue or flavivirus oligonucleotide primer pair followed by a second amplification with serotype-specific oligonucleotides. These procedures also vary in the genomic location of primers (E, NS1, E/NS1, prM/E, NS5, NS5/3′), and in their specificity and sensitivity. Some protocols allow the detection of less than 50—100 dengue virus pfu.[118–135] In the Americas, the protocol developed by Lanciotti et al.[122] has been widely applied. These investigators designed consensus primers to C/prM genes that amplify a

511 bp product. In a second round of PCR, using type-specific primers, DNA products of different sizes are amplified, allowing the differentiation of serotypes. Modifications to this protocol as well as new protocols have also been used.[120,125,130,134,135]

When correctly applied, PCR has remarkable advantages as a tool for dengue diagnosis. The use of PCR allows the detection of dengue in stored samples over long periods. Alvarez et al[136] and later Sariol et al[29] demonstrated the dengue 2 virus in serum and tissue autopsy samples from DHF/DSS cases that had been stored for more than 15 years. In addition, they were able to classify the isolates by genomic sequencing. Alternatively, different authors have applied PCR to entomological surveillance. Chow et al., 1998 and Kow et al. in 2001[137,138] identified dengue viruses in *Aedes aegypti* and *Aedes albopictus* mosquito pools during a one-year surveillance period in Singapore, allowing the application of vector control measures. Infected *Aedes aegypti* were detected as early as six weeks before the recognition of a dengue outbreak in 1995 and 1996. These authors recommend this method as an early warning monitoring system for dengue outbreaks.

PCR also allows the detection of concurrent infections by multiple serotypes both in serum samples and in isolates from tissue culture or mosquito inoculation.[139–141] Laille et al., 1991, were able to detect dual viremia by dengue 1 and dengue 3 in six DF patients during the 1989 New Caledonia epidemic.[139] Similarly, Loroño-Pino et al. were able to show that 5.5% of the 292 samples they tested showed evidence of concurrent dengue infection with two or more serotypes.[141]

One of the most important applications of PCR is the study of genetic strain variability in order to identify the origin of epidemics and reveal markers of virulence. In conjunction with nucleotide sequencing or restriction enzyme analysis, PCR has allowed the classification of dengue serotypes into genotypes (Table 2).[142–150] Rico-Hesse and co-workers have developed a method to compare dengue 2 genomes directly from patient plasma. They found some amino acid and nucleotide changes in the E protein gene as well as within the untranslated region that may represent primary determinants of DHF.[151,152] In other studies, Kuno et al. established the genetic relationship among viruses of the *Flavivirus* genus. They proposed that two branches of the virus evolved from the putative ancestor — non-vector and vector-borne virus clusters — and from the latter cluster emerged tick-borne and mosquito-borne viruses.[153] Recent reports suggest that intra-serotype recombination of dengue virus occurs.[154–157] The consequences of these findings are not well defined.

Finally, new PCR protocols and methodologies have appeared that allow the rapid detection and the quantification of RNA. In 1999, Laue et al. applied a fully automated amplification protocol (based on the TaqMan principle) to measure virus-specific DNA amplification. This method demonstrates a high specificity and sensitivity; it eliminates the possibility of cross-contamination and allows the determination of viral load.[158] More recently, Callahan et al., 2001, applied a TaqMan RT/PCR assay to identify dengue serotypes and groups, reporting a sensitivity of 92.5% and 98.5% respectively, and a specificity of 100%, when compared to viral isolation in C6/36 cells. Results were obtained in less than two hours.[159] Wu et al., 2001, applied an isothermal nucleic acid sequence-based amplification (NASBA) assay to amplify the four dengue serotypes using a set of universal primers and serotype-specific capture probes for typing. Nucleic acid was amplified without thermo cycling and the product was detected by probe hybridization using electrochemiluminescence. The assay

**Table 2** Dengue genotype classification according to different authors.

| | Group or subtype* | References |
|---|---|---|
| Den 1 | I French Polynesia/Fiji/Singapore/Indonesia/Nauru/New Caledonia/Tonga; II Jamaica/French Guyana/New Caledonia/Brazil/Mexico/Aruba/Cuba/Peru/ Nicaragua/Thailand/Senegal/Malaysia/Puerto Rico; III Philippines/Thailand | 146 |
| Den 2 | I Puerto Rico/Tahiti/Tonga/Colombia/Mexico/Venezuela/Trinidad; II Taiwan/Philippines/New Guinea/Thailand; III Vietnam/Thailand/Jamaica; IV Indonesia/Seychelles/Burkina Faso/Sri Lanka; V Ivory Coast/Burkina Faso/Senegal | 142,151 |
| Den 3 | I Philippines/Malaysia/Indonesia/Tahiti/Fiji; II Thailand; III Sri Lanka/Samoa/India/Mozambique; IV Puerto Rico/Tahiti | 145 |
| Den 4 | I Thailand/Philippines/Sri Lanka; II Tahiti/Puerto Rico/Brazil/New Caledonia/El Salvador/Mexico/Dominica/Indonesia | 147 |

* Genomic classification in group or subtypes based on the nucleotide envelope study.

showed a sensitivity of 98.5% and a specificity of 100% when compared to viral isolation from the C6/36 cell line.[160] More recently, a biosensor has been added to the NASBA technique allowing the rapid detection of dengue virus RNA in only 15 minutes.[161] In an alternative approach, a fluorogenic RT-PCR system was developed for the quantification and identification of dengue viruses using conserved and serotype-specific 3′-non-coding sequences. This system is able to detect 20—50 pfu/ml of serum and showed a sensitivity of 92.8% and specificity of 92.4% compared to virus isolation in cell culture.[162]

## Laboratory case classification

The use of these diagnostic tests for dengue allows the classification of suspected clinical cases to become probable or confirmed cases. A positive IgM serology, or a reciprocal HI antibody $\geq$1280 or equivalent IgG titer by ELISA are the criteria indicating probable dengue infection. Dengue virus isolation, dengue antigen demonstration or positive PCR, and a fourfold or greater change in reciprocal IgG in paired sera, are used to confirm dengue infection. Both probable and confirmed cases are reportable to health authorities.[7]

## Dengue diagnosis, where we are today? The main problems

IgM capture ELISA, virus isolation in mosquito cell lines and live mosquitoes, dengue-specific monoclonal antibodies, and PCR have all represented major advances in dengue diagnosis. However, some problems still warrant the timely development of new solutions:

- Virus isolation is time consuming.
- PCR requires specific laboratory equipment and facilities as well as extensive evaluation of the different protocols under field conditions.
- IgM antibody detection requires proper timing and is confounded by false positive reactions and the long persistence of IgM antibodies, commercial kits still need to be critically evaluated, and the costs and availability of these kits and other reagents need to be addressed.

## What is still needed for a better diagnosis?

The continued development of inexpensive, sensitive, specific and easy tests that allow for early dengue diagnosis are still needed. Specifically, the following aspects require the greatest attention: To develop:

(a) Tests for early clinical diagnosis of individuals.
(b) Serological tests able to differentiate dengue from other flavivirus infections and even more specifically to determine the infecting dengue serotype.
(c) Easy and inexpensive protocols for genomic characterization and viral load, including those that can be applied in the field.
(d) Modifications of existing protocols that simplify specimen handling and transportation.
(e) Recombinant antigens as tools for test evaluation and to produce these for serological tests.
(f) Tools that can suggest a prognosis, allowing for better management of clinical follow up.

In addition to these specific items it is also necessary to implement mechanisms for greater reagent availability, for sharing standard reagents (antigens, monoclonal antibodies, cell cultures, positive and negative control sera), for the standardization of protocols in endemic regions, for improving the quality and quantity of the proficiency test, and for the enhanced exchange of information and experiences between endemic areas including the development of collaborative research projects.[163] The role of World Health Organization's Collaborating Centers in all these aspects is crucial.

There are some other problems and needs that are not specifically related to technological development. For instance, the laboratory infrastructure, technical expertise, and research capacity is limited in many countries where dengue is endemic. These factors negatively influence dengue surveillance, clinical management of cases, and the development of new approaches to dengue control. Although there are no published estimates of the financial support allocated for dengue field activities and research, it is not disputed that funds are scarce. It is urgent that funds be mobilized to increase basic public health capacity and improve the infrastructure in endemic countries. This could result in a decrease in dengue morbidity and mortality, improved control, disease prevention and improved knowledge of dengue and DHF/DSS. The result would be an increase in investigations directed towards diagnosis, vaccine development and pathogenesis. Finally, we must advocate for the urgent development of an appropriate rapid, early and accessible diagnostic method for dengue in order to save lives.

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