LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTIONS

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Abstract: Accurate and efficient diagnosis of dengue is important for clinical care, surveillance support, pathogenesis studies, and vaccine research. Laboratory diagnosis is also important for case confirmation. Here is presented a review of the current state of dengue virus diagnosis. Laboratory dengue diagnosis can be performed through virus isolation, genome and antigen detection and serological studies. For virus detection, dengue viremia is short, usually observed two or three days before onset of fever and lasts four to five days later. Therefore, samples for virus detection must be taken in the first four to five days of disease during the febrile phase. In recent years, PCR (polymerase chain reaction) has become an important tool as a quick method for diagnosis of dengue. Another method of diagnosis is detection of NS1 antigen, using a commercial ELISA kit. Although PCR is the most sensitive and rapid method for the detection of dengue virus in early stage of disease, classical dengue virus culture using dengue tissue culture seeds also has important benefit, especially for genotype studies. In serologically based diagnosis of primary infection, the dominant immunoglobulin isotype is IgM. Anti-IgM may appear during the febrile phase in 50% of cases, in the other half, it appears within 2-3 days following defervescence. Once detectable, IgM levels rise quickly and appear to peak about 2 weeks after the onset of symptoms, and then they decline to an undetectable level over 2-3 months. Anti-IgG appears shortly afterwards at a very low level. The physiological definition of a primary infection is therefore characterized by a high molar fraction of anti-dengue IgM and low molar fraction of IgG. Secondary dengue infections are characterized by a rapid increase in IgG antibodies and when anti-dengue IgM appears in most instances, the levels are dramatically lower.

Keywords: dengue infection, dengue virus, laboratory diagnosis

INTRODUCTION

Dengue virus (DENV) consists of four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) that display a high degree of antigenic cross-reactivity with each other as well as with other mosquito and tick-borne flaviviruses such as Japanese encephalitis (JE), yellow fever, West Nile, and tick-borne encephalitis viruses (Calisher *et al,* 1989; Innis, 1995). In countries where dengue is now or is becoming endemic, the cocirculation of two or more flaviviruses such as dengue and JE in Asia, dengue and JE and West Nile in India, and dengue and yellow fever in the Americas makes the serologic diagnosis of acute DENV infection a difficult task. From a public health and clinical care perspective, the accurate diagnosis of DENV infection as well as the identification of circulating DENV serotypes affects public health policy and action for vector control as well as individual case management.

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By 1964, dengue fever (DF) and dengue hemorrhagic fever (DHF) were recognized as a serious public health problem in most of Southeast Asia. Hammon and Sather (1964) summarized the difficulties of making a serotype-specific diagnosis due to the serologic cross reactivity between dengue and other flaviviruses. Three assays were available in 1964 to distinguish DENV serotypes: virus neutralization using a suckling mouse model (Sabin, 1950), complement fixation (Casals, 1949) and hemagglutination inhibition (HI) (Clarke and Casals, 1958). The latter two methods were plagued with a high degree of serologic cross-reactivity, and the former complicated by the use of suckling mice. By 1990, several milestones in dengue diagnosis were achieved, to include the use of mosquitoes to propagate DENV, the development of mosquito cell lines for virus isolation, monoclonal antibodies to all four DENV serotypes, the adaptation of the enzyme immunoassay (EIA) format to dengue diagnosis, and the first genome-based diagnostic efforts (Shope, 1990).

The purpose of this chapter is to detail assays currently available to diagnose DENV infection. Understanding a diagnostic assay's advantages and limitations is essential to proper interpretation and application whether that application is at the level of the patient or population.

ANTIBODY AND VIRUS PATTERNS IN DENGUE VIRUS INFECTION

Upon the bite of an infected mosquito, DENV is introduced intradermally and replicates within skin dendritic cells (Wu *et al,* 2000). From the time of inoculation, DENV replicates and disseminates through the lymphatic system producing measurable viremia approximately 3 days after inoculation lasting, approximately another 4 days (Fig 1). The onset of fever and symptoms occurs approximately 24 hours after the onset of measurable viremia. Fever will last 4 days on average followed by a sudden defervescence (Chandler and Rice, 1923; Siler *et al,* 1926; Simmons, 1931; Sabin, 1955; Kalayanarooj *et al,* 1997; Vaughn *et al,* 1997). The day of defervescence is an immunologically important landmark in the course of DENV infection as it defines the approximate onset of plasma leakage in patients with DHF (WHO, 1997; Green *et al,* 1999).

Primary dengue

Primary DENV infection occurs when a patient lacking previous exposure to a flavivirus develops an acute DENV infection that results in dengue-specific antibody production. The patient with a primary infection rarely develops DHF and anti-dengue antibody evolves slowly during the course of the clinical illness with high production of IgM antibody (Vaughn *et al,* 1997). The molar ratio of IgM to IgG is high (≥1.8:1.0) for at least three weeks following infection (Innis *et al,* 1989). Using HI and neutralizing antibody, a primary infection is defined by low titers of antibody that develop slowly (Russell *et al,* 1967; WHO, 1997).

Secondary dengue

Secondary dengue implies previous flavivirus exposure (secondary flavivirus infection) or a previous DENV infection with a different serotype (secondary DENV infection). The IgG antibody response occurs early and vigorously during the clinical illness; nearly all patients have diagnostic levels of antibody by EIA within 24 hours of defervescence (Innis *et al,* 1989; Vaughn *et al,* 1997). The rapid increase in antidengue antibody to high levels indicates an anamnestic (memory) response (Halstead *et al,* 1983). The dengue-specific IgM response is more variable with a IgM to IgG ratio ≤1.8 as measured by IgM capture ELISA (Innis *et al,* 1989).

The level of IgM response may relate to the number of new epitopes present on the current infecting virus compared to the previous flavivirus. That is, previous infection with a more distantly related flavivirus (*eg*, JE virus) will result in higher IgM levels by EIA when infected by a DENV than if the current infection follows a previous DENV infection with another serotype. HI and neutralizing antibody levels elevate quickly to high titers in secondary infections (HI titer ≥1:2560) (WHO, 1997). Again, the degree of shared epitopes may influence the degree of anamnestic antibody response.

Viremia pattern

Due to antibody cross-reaction, definitive diagnosis of DENV infection requires isolation of the virus or detection of virus genome. The pattern of viremia in dengue patients correlates closely with fever with peak levels (titers up to 10 logs/ml) occurring 2 to 3 days following the onset of illness, which is typically 2 to 3 days prior to defervescence (Vaughn *et al,* 2000). For DENV-1, DENV-2, and DENV-3, the viremia titer correlates with dengue disease severity (Vaughn *et al,* 2000; Libraty *et al,* 2002). This finding is of more than theoretical interest as it provides a potential tool for the clinician to assess the risk of severe disease (DHF) prior to the onset of plasma leakage.

With the development of new and rapid tools to measure viremia titer, this may prove to be a potentially important measurement in defining persons at risk for DHF. Viremia level, as originally determined by Vaughn and colleagues (2000), used serial titrations of viremic plasma from dengue patients inoculated into *Toxorhynchites splendens* mosquitoes. The titer at which infection occurred in half of the *T. splendens* was determined using probit analysis resulting in the mean infectious dose 50% (MID_{50}). This is a time consuming and tedious process requiring serial specimen dilution and numerous mosquito inoculations followed by a two-week incubation period and virus detection in each mosquito by indirect immunofluorescence or RT-PCR.

New molecular tools are proving useful to measure dengue viral load (Houng *et al*, 2001; Libraty *et al,* 2002). However, where mosquito or cell culture inoculation measures viable virus, molecular approaches detect only the presence of genome. RT-PCR methodologies may allow a virus-based diagnosis extending many hours past the detection of replicating virus due to its detection of viral genome in virus-antibody complexes (neutralized virus) (Wang *et al,* 2003). How rapid molecular diagnostic tool will affect the clinical management of DHF patients needs to be determined in prospective studies.

DIAGNOSTIC PATHWAY IN PATIENTS WITH SUSPECTED ACUTE DENGUE

Fig 1 illustrates the diagnostic options currently available to diagnose an acute DENV infection

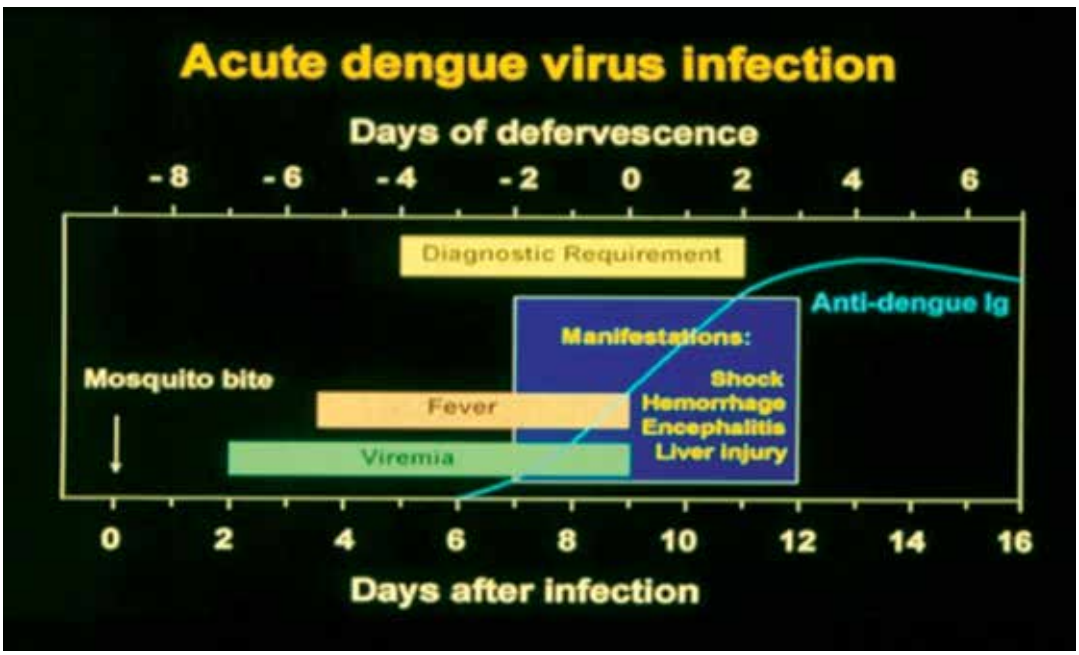

Fig 1–Diagnostic pathway in patients with suspected dengue.

in patients with suspected dengue. Diagnostic options are divided first into assays that detect the presence of the virus (including virus antigen and virus genome) versus assays that detect the host's response to the virus (antibody). Virus detection methods can be divided between time-consuming virus culture methods using animals, mosquitoes, or cell culture versus rapid antigen or genome detection methods. Likewise, antibody detection methods include assays that can be completed in a few minutes or require a week or more to ascertain.

The utilization of multiple approaches with properly timed specimens assures diagnosis in virtually every case of suspected dengue. This diagnostic rigor is required to conduct research in dengue pathogenesis. Is this type of diagnostic breadth needed for every laboratory to be made available to every clinician? Ideally, every country experiencing or at risk for dengue epidemics should have at least one center of excellence that is proficient to isolate virus, detect genome, and to carefully characterize antibody responses. This would help to better understand the depth and breadth of the dengue pandemic.

The capacity to diagnose clinically similar outbreaks such as leptospirosis is also important. Serotype specific virologic diagnoses over time may someday allow the prediction of the timing and severity of dengue epidemic in order to allow vector control efforts to prevent or abate such epidemics.

For the clinician, a hospital-based laboratory that can perform basic dengue serology can quickly confirm a dengue outbreak. However, since antibody-based assays do not routinely become positive until the danger period for DHF has passed, the diagnosis is of less value to the patient who is recovering. To have an impact on the care of an individual patient, a reliable rapid molecular diagnosis is needed to assist in the differential diagnosis and to reduce morbidity and mortality. For suspected dengue patients, serum specimens should be collected early in the febrile phase and stored at -70°C for virus isolation/detection and at -20°C for acute phase serology to be paired with a specimen drawn at least 7 days later for convalescent serology (WHO, 1997).

The practice in many dengue hyperendemic countries is to draw blood upon admission and at the time of discharge one to two days following defervescence. In this setting, most dengue patients are experiencing secondary infections and the serology will be positive soon after defervescence. For patients with primary infections, a subsequent follow-up specimen is needed to confirm or rule out DENV infection if attempts at virus isolation or identification fail.

For travelers returning to the United States with suspected dengue, acute and convalescent samples can be sent to the state health department with a request to forward them to the CDC for testing.

SEROLOGICAL ASSAYS

Hemagglutination inhibition (HI) assay

The path towards the serologic diagnosis of dengue started in 1950, when Sabin (1950) discovered that arboviruses in general and dengue virus (DENV) in particular, are able to agglutinate certain types of erythrocytes. Normal erythrocytes in a suspension will settle to the bottom of a test tube or well and form a compact, dense button of red blood cells after 30 minutes to one hour. Agglutination of red cells using virus antigen will prevent this normal settling to occur and results in a uniform lattice of cross linked cells covering the lower part of the tube or well (hemagglutination). Casals and Brown (1954) modified the technique using acetone and ether to extract (purify) the hemagglutinating antigens (HA) and developed a hemagglutination inhibition (HI) assay. In this assay, DENV specific antibody binds the HA preventing lattice formation allowing the red blood cells to clump to the bottom of the test well (Fig 2).

Test serum could be tittered to determine when hemagglutination was no longer inhibited. Porterfield (1954, 1957) and Clarke and Casals (1955) further improved this assay by using female goose red blood cells rather than human cells to remove nonspecific inhibitors of hemaglutination. The techniques used for hemagglutination and HI as described by Clarke and Casals in 1958 have

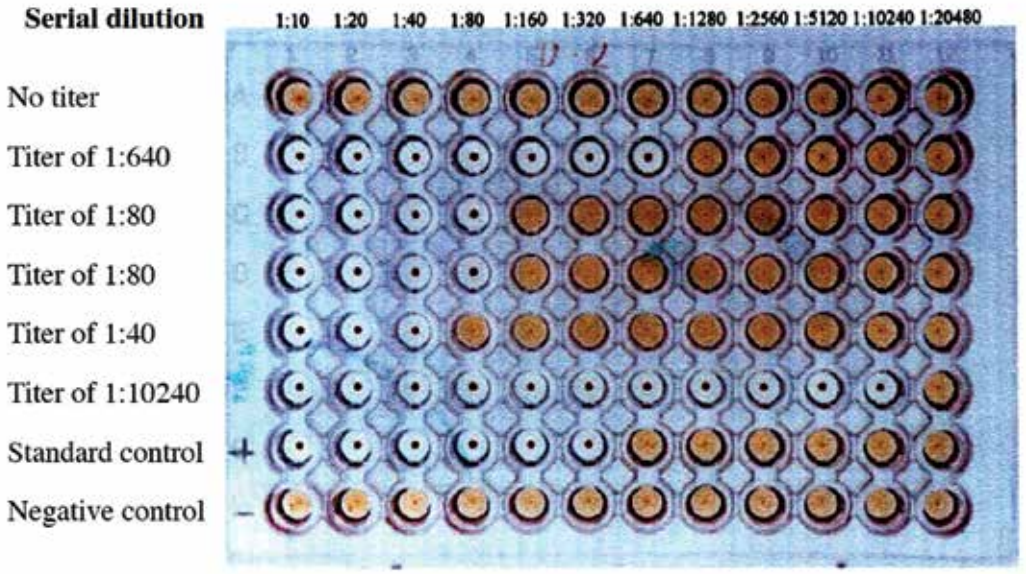

Fig 2–Hemagglutination inhibition assay.

remained largely unchanged today apart from adaptation to microtiter plates in 1980 and remains a fundamental tool to diagnose acute DENV infection and in seroprevalence studies (WHO, 1997).

Agglutination of red cells is dependent on pH and the amount of HA present. HA is quantified as a titer where an HA unit of 1 represents the highest dilution which causes hemagglutination of red cells. An additional 1 HA unit is thus given for each subsequent lower titer of antigen. For example, if the highest titer of a DENV-2 antigen causing hemagglutination was found to be 1:160 and by convention given an HA unit of 1 at this titer, 2 HA units would be a titer of 1:80; 3 HA units for 1:40; 4 HA units for 1:20 and 5 HA units for 1:10. The hemagglutination assay allows quantification and standardization of dengue antigen (HA) produced from a variety of sources with the most common sources being suckling mouse brain and cell culture.

As until recently practiced in the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, 1-to-2 day old suckling mice (*Mus musculus*) are intracranially inoculated with 0.02 ml of a dengue serotype specific virus suspension. Mice are observed twice a day for the first signs of encephalitis (failure to eat as evidenced by lack of milk in the stomach, color change, wasting, or tremors), which occurs 3-to-10 days after inoculation. For DENV-1 this usually occurs at day 5, day 4 for DENV-2, day 7 for DENV-3, day 4 for DENV-4, and day 3 for Japanese encephalitis virus. Harvested brain is made into a 20% (weight/volume) suspension in an 8% sucrose solution and homogenized. The homogenate is then acetone extracted, condensed, washed and resuspended in sterile normal saline. All antigen is assayed for the number of HA units.

The hemagglutination assay itself is performed best using goose red blood cells (RBCs) though RBC from other species can be used. Goose RBCs can be obtained from adult female geese (*Anser cinereus*) and should immediately be placed in Alsever's solution (glutaraldehyde-fixed goose RBCs may also be used) (Wolff *et al,* 1977). Goose RBC's are then washed with dextrose-gelatin-veronal solution and are brought up to a final 8% solution just prior to assay use. Buffers of differing pH have been found to be optimal for the agglutination of different flaviviruses and specific to each dengue serotype: pH of 6.2 for DENV-1; 6.4 for DENV-2; 6.6 for DENV-3; and 6.8 for DENV-4. It is essential

that the RBCs be suspended in a buffer set at a specific pH for each dengue serotype prior to use in the assay.

To prepare the test sera, non-specific inhibitors must be removed (that is, some human serum can inhibit agglutination in the absence of measurable dengue antibody). This is done using acetone, ether or kaolin prior to performing the HI assay (Monath *et al,* 1970). Diethylaminoethyl-Sephadex (DEAE-Sephadex) has also been used with success (Altemeier *et al,* 1970). At AFRIMS, acetone extraction is the preferred method and is performed by adding heat-inactivated serum to cold acetone and decanting the mixture. A drop of goose red blood cells is added to the sera, mixed and removed to eliminate non-specific agglutination of erythrocytes.

The HI assay at AFRIMS laboratory is performed in 18 x 10 v-shaped well Lucite plates that are clearly marked for each serum and dilution. Two-fold serial dilutions of the test sera and standard positive and negative controls are placed into each well using a 0.025 ml loop followed by the addition of 0.025 ml of 8 to 16 HA units of specific dengue serotype antigen and the plates are covered and incubated at 4°C overnight. Ideally, all sera from a single patient should be tested in the same assay. As a screening test, two broadly cross-reactive dengue antigens may be used (DENV-2 and DENV-3) with only slight loss of sensitivity. The following morning the test plates are allowed to reach room temperature and 0.05 ml of an 8% goose red cell stock solution diluted 1:24 in the proper pH buffer is added to each well. The plate is allowed to sit undisturbed for one hour at room temperature and the wells that have or have not agglutinated are recorded. The HI titer is taken as the highest serum dilution that causes complete inhibition of agglutination (Fig 2). The interpretation of dengue HI antibody titer is based on WHO criteria (WHO, 1997). Work by Burke and colleagues suggest a good correlation between HI and PRNT50 in paired specimens collected 7 months apart (Burke *et al,* 1988).

The primary sources of inter-assay variation for the HI assay are: 1) the amount and quality of HA (appropriate amount varies by serotype), 2) pH (varies by serotype), and 3) interoperator variation to interpret agglutination in each well. Sources of assay variation can be reduced by: establishing HA units for stock dengue antigen and periodically checking to ensure that antigen HA activity is not lost, strictly following guidelines on the proper pH for each dengue serotype antigen, running both positive and negative serum controls on each plate, testing paired sera on the same plate, and reading the plate on a white background or a low-light fluorescent light box.

Interpretation of results is relatively straightforward (Table 1). For suspected acute dengue, the timing of the collection of paired sera is important. Sera obtained 7 days or more apart should demonstrate a four-fold rise in titer if the patient was acutely infected with DENV: Serum collected a week apart will often require the patient to be seen in follow-up; a problem for many clinics. If serum is collected less than 7 days apart and a 4-fold rise in titer is not seen; the result must be read out as non-diagnostic. If there is a 4-fold increase and the titer rises to 1:2560 or higher, it indicates an acute secondary flavivirus infection. If the end titer is 1:1280 or less, the interpretation is an acute primary DENV infection. For many patients with a secondary DENV infection, a 4-fold increase will be seen well before 7 days. If a single specimen or paired specimens show a titer of 1:2560 or higher without a 4-fold increase, the interpretation is a recent DENV infection, possibly acute or having occurred during the previous couple of months.

Importantly, the HI test fails to discriminate adequately between the closely related flaviviruses such as dengue, JE, and West Nile. This could produce results that are difficult to interpret in countries where these viruses co-circulate. Despite these limitations, the HI assay is a powerful technique that is still a standard assay for seroprevalence studies as well as in the diagnosis of acute primary and secondary DENV infections. The HI assay is a robust assay that can be performed with minimal laboratory equipment, reagents and expense. Equally important is that the reagents can be made locally and are readily sustainable for the developing dengue diagnostic laboratory.

Table 1. World Health Organization criteria to interpret dengue hemagglutination inhibition assay results.

Change in antibody titer	Sample interval	Antibody titer at time of convalescence	Interpretation
≥ to a 4-fold rise	Paired sera with ≥7 days of separation.	≤1:1280	Acute primary flavivirus infection.
≥ to a 4-fold rise	With any specimen.	≥1:2560	Acute secondary flavivirus infection.
≥ to a 4-fold rise	Paired sera with <7 days of separation.	≤1:1280	Acute flavivirus infection, indeterminate primary/secondary.
No change in titer	With any specimen.	≥1:2560	Recent secondary flavivirus infection.
No change in titer	Paired sera with ≥7 days of separation.	≤1:1280	Not dengue.
No change in titer	Paired sera with <7 days of separation.	≤1:1280	None
Uncertain	One sera specimen.	≤1:1280	None

Source: WHO, 1997.

Plaque reduction neutralization assay (PRNT)

Dulbecco (1952) described a chick embryo fibroblast plaque assay for several viruses including Western equine encephalitis and Newcastle disease viruses. This technique allowed quantification of viruses *in vitro* as plaque forming units (PFU). In 1959, Henderson and Taylor described a method to detect arbovirus viral plaques in an agar overlay stained with neutral red and demonstrated its utility to measure serum antibody neutralization titers. The standard neutralization test prior to the availability of tissue culture was the suckling or weanling mouse neutralization test, usually performed using a constant serum dilution mixed with log dilutions of virus prior to administration to the test animals and monitoring for illness. The ability of the serum to neutralize was calculated as the log neutralization index. The DENV neutralization assay using suckling mice in a challenge virus resistance assay was adapted to BS-C-1, PS and LLC-MK2 cell lines by Halstead *et al* (1964) and Sukhavachana *et al* (1966).

It was not until 1967 however, that a direct *in vitro* assay to measure DENV neutralizing antibody and DENV identification by serology was developed by Russell *et al* (1967). This assay became known as the dengue plaque reduction neutralization test (PRNT) and utilized prototype dengue viruses, monkey sera controls, LLC-MK2 cell lines and an agar overlay media with neutral red staining. A probit analysis was used to measure plaque reduction as the serum titer required to reduce dengue viral plaques by 50% ($PRNT_{50}$). This technique introduced an efficient and reproducible assay to measure dengue serotype specific neutralizing antibody and became the standard assay to measure dengue antibody.

Variations of this technique were introduced thereafter, such as a micrometabolic inhibition test using BHK-21 cells and a microculture plaque-reduction test utilizing the LLC-MK2 cell line (Sukhavachana *et al,* 1969), microplate cultures using BHK-21 cells (Fujita *et al,* 1975), a focus reduction method using peroxidase-anti-peroxidase staining of BHK-21 cells (Okuno *et al,* 1978), a semi-micro method in LLC-MK2 cells using a 70% plaque reduction criteria (Morens *et al,* 1985a), and a simplified PRNT assay using BHK-21 cells (Morens *et al,* 1985b). These assays are being used to determine serological responses to dengue vaccines

(Jacobs and Young, 2003), in seroepidemiologic studies defining outbreaks of DENV (Halstead *et al,* 2001) and in pathogenesis studies of dengue shock syndrome (Green *et al,* 1999).

The microneutralization assay (Vorndam and Beltran, 2002) is based on the same principle as PRNT; however, instead of counting the number of plaques per well, this assay uses a colorimetric measurement of virus-induced cell lysis to determine the end-point dilution.

Enzyme immunosorbent assays (EIA)

Innis and colleagues in 1989 applied the anti-JE IgM antibody capture EIA of Burke and Nisalak (1982) to dengue and developed a serological assay to diagnose acute dengue in countries where JE and dengue viruses co-circulate (Innis *et al,* 1989). This assay allows the rapid (within 48 hours) diagnosis of acute DENV infection. Equally important, this assay was optimized to distinguish primary from secondary dengue on the basis of IgM/IgG ratios and by running a concomitant IgM capture JE ELISA, eliminating the false positives that might occur from acute JE virus infections. This assay has served as a standard to measure newer assays (Vaughn *et al,* 1999).

The ELISA assay is widely used in the diagnosis of viral pathogens due to the relative ease to set up the assay in a 96-well format, its high degree of reproducibility and for the option to use automatic plate washers and scanners. In the Innis assay (Innis *et al,* 1989), anti-dengue isotypecapture enzyme immunoassays measure the proportion of immunoglobulin isotype reactive with dengue or JE antigen. Briefly, the method uses 96 well plates sensitized overnight with either goat anti-human IgM or IgG antibody. Control and test sera are diluted 1:100 and placed in the wells overnight. IgM or IgG is then captured onto the respective plates and followed by tetravalent dengue antigen (16 HA units each of DENV-1, DENV-2, DENV-3, and 8 HA units of DENV-4) for the dengue EIA or JE antigen (50 HA units) for the JE EIA. This is followed by an anti-flavivirus horseradish peroxidase conjugate with substrate producing a quantitative colorimetric result read by an EIA plate reader. A binding index (81) is calculated using the optical density (0D) of the test sample minus the OD of the negative control all divided by the OD of the weak positive control minus the OD of the negative control. The 81 multiplied by 100 generates EIA units where ≥40 units is considered positive for the IgM capture assay. The sensitivity of diagnosing acute dengue is 78% on admission sera and 97% in paired sera with a specificity of 100%. The value of this assay in addition to diagnosing acute dengue is in distinguishing between acute dengue versus acute JE. A ratio of anti-dengue IgM to anti-JE IgM of ≥1.0 is typical of acute dengue whereas a ratio ≤1.0 is consistent with acute JE virus infection.

The added value of this assay is that it distinguishes primary from secondary DENV infection. Sera defined as consistent with primary or secondary dengue by HI titers established ratio cut-offs for IgM/IgG where a ratio of IgM to IgG units ≥1.8 is consistent with primary DENV infection and a ratio of <1.8 is consistent with secondary dengue. Dengue EIA, when compared to HI using WHO standards, demonstrated a high coefficient of correlation and agreement rate (Chungue *et al,* 1989; WHO, 1997).

As the format of this assay has become widely used in the diagnosis of acute dengue, it is worthwhile to discuss some of the quality control aspects of this assay and the methods employed to decrease inter and intra-assay variation. The primary sources of inter-assay variation for this assay are: 1) the dilution of the anti-flavivirus IgG enzyme conjugate; 2) duration of the chromogen-substrate reaction; and 3) the plate coating sensitization step (amount of anti-isotype antibody bound). Samples and antigens used in this assay are used in quantities that saturate the capture system therefore the key limiting components of the assay are the bound anti-isotype antibody and the IgG-enzyme conjugate. The amount of bound anti-isotype antibody is affected by the quantity used, the duration of sensitization, and the type of plates used.

Reduction of variation in the sensitization step can be accomplished by consistently using plates

that were used to optimize the assay and to pre-sensitize plates in batches and store at -20°C. Plates can be kept at -20°C for up to one month without significant loss of bound anti-isotype antibody. It is also important to determine the assay dilution of the anti-flavivirus IgGHRP conjugate. This is accomplished by determining the assay dilution that yields an OD at 492 nm of 0.4 (established cut-off for a positive test) when a 1:100 dilution of the weak positive control is used with test sera. Intra-assay variation should be 10% or less. Within one complete assay, all four positive standards should be in the OD range of 0.25 to 0.55. Values above this range result in a decrease in the assay's sensitivity and values below this range will result in a decrease of specificity.

EIAs using cell culture derived dengue antigens and those using dengue monoclonal antibodies rather than patient-derived control serum have been found to be as sensitive and specific as the Innis assay (Lam *et al,* 1987; Kuno *et al,* 1991; Cardosa *et al,* 1992). Additionally, commercial alternatives are now available and compare favorably with the original EIA described by Innis and colleagues (Lam *et al,* 1996; Cuzzubbo *et al,* 1997; Sang *et al,* 1998; Vaughn *et al,* 1999; Wu *et al,* 1999; Cuzzubbo *et al,* 2000; Groen *et al,* 2000; Lam *et al,* 2000). The dengue IgM and IgG capture EIA assay (PanBio Dengue Duo) demonstrated a sensitivity of 99% and specificity of 92% compared to the Innis assay (Vaughn *et al,* 1999).

A variation of the traditional serum based form of the EIA has recently been developed using saliva for the diagnosis of acute infection (Cuzzubbo *et al,* 1998; Artimos de Oliveira *et al,* 1999). Saliva contains both dengue-specific IgM and IgG that can be measured in a traditional IgM capture EIA format. One prospective study using the PanBio Dengue Duo ELISA determined a sensitivity of 92% and a specificity of 100% compared to the serumbased Innis EIA (Cuzzubbo *et al,* 1998).

The dengue IgM/IgG EIA allows high throughput and reagents are commercially available or readily available from reference laboratories. The advantages of the anti-dengue IgM and IgG isotype-capture enzyme immunoassay are its ability to detect elevated levels of IgM in samples collected during the acute period, an interval when most DHF patients are still hospitalized; that is, a single specimen positive for dengue IgM indicates acute dengue. However, it may take until 3 days post-defervescence before all dengue patients test-positive by EIA. Serum contains no inhibitor of the EIA, therefore no pre-treatment is needed; and the assay can be optimized to distinguish between different flaviviruses.

A further advantage of the EIA is its ability to detect IgM in the CSF in cases where encephalitis is suspected or saliva to minimize venipuncture in prospective field studies. Commercially available EIA kits have demonstrated high levels of sensitivity and specificity for acute DENV infection making this diagnostic assay a key tool for the dengue diagnostic laboratory.

Indirect fluorescent antibody (IFA) test

This assay to detect dengue specific IgM and IgG using fluorescent antibody is used primarily in research laboratories. Vathanophas and colleagues developed it in 1973 (Vathanophas *et al,* 1973). The assay utilizes a solid phase (usually dengue virus infected cells that are cold acetone fixed onto slides) that binds human dengue antibody, which in turn is detected by fluorescein-conjugated anti-human antibody. Visible fluorescence seen on the slides constitutes a positive antibody test. Serial dilutions of test serum are used to measure the amount of antibody present as an antibody titer. This method is limited due to its time-intensiveness, subjective reading, reliance on infected cells as the antibody capture agent and lack of automation. The major advantage of this assay is its relative low cost to perform with a few samples (Boonpucknavig *et al,* 1975).

Rapid antibody assays

In 1988, Cardosa and colleagues reported a simplified dengue assay that could be used in less developed laboratories with the potential to be used in the field (Cardosa *et al,* 1988). The dot enzyme immunoassay (DEIA) was more sensitive than the HI test and had the advantage of using a

single serum dilution as a cutoff point to diagnose acute DENV infection. This and similar assays are an extension of the western blot where DENV proteins are transferred onto a solid phase paper or dipstick and upon exposure to DENV-specific antibody and an anti-human antibody detection system, whereby a band appears indicating a serological response to DENV.

These dot enzyme immunoassays (DEIA) or dot-blot tests offer a diagnostic tool that is rapid (usually 4-6 hours or less), able to detect both IgM and IgG dengue antibody and hence acute primary or secondary DENV infection, and require minimal expertise or laboratory equipment (just a centrifuge and water bath). In the dot blot assay of Cardosa and colleagues, strips containing DENV antigen were incubated with human sera which allows the binding of dengue-specific antibody to the DENV antigen on the test strip. A biotinylated anti-human IgG, or IgM, depending on the assay, detected the bound dengue antibody and after a signaling reaction, displayed a color band on the test strip. A positive IgM alone signified an acute primary DENV infection and both a positive IgM and IgG indicated an acute secondary DENV infection.

Another example of this approach was the Dengue Rapid Test developed by PanBio, LTD (Lam and Devine, 1998; Vaughn *et al,* 1998). In less than 7 minutes, this immunochromatographic test detected dengue-specific IgM and IgG antibodies. Compared to standard diagnostic techniques and using hospital admission and discharge sera, this test demonstrated 100% sensitivity to diagnose DENV infection and reliably distinguish primary from secondary DENV. This assay has migrated towards the use of recombinant antigens and a dipstick format (Wu *et al,* 1999; Cuzzubbo *et al,* 2001; Parida *et al,* 2001). Additional rapid assays are being developed and coming to the market (Groen *et al,* 2000). The limitations of rapid antibody-based assays are that specificity is decreased due to the cross-reactive nature of antibody to other flaviviruses such as JE virus and its dependence on the appearance of IgM, which can appear late in the course of infection or be blunted during secondary DENV infections.

VIRUS DETECTION

Intracerebral inoculation of suckling mice

DENV was first isolated in August 1942 during an epidemic of dengue that occurred in the Nagasaki-Sasebo region of Japan (Hotta, 1952). Blood taken from patients within 48 hours after the first rise of temperature were inoculated intracerebrally (IC) into suckling white mice. Infected mice developed debility, tremors, and paralysis occurring in the hind legs. Since this first description, IC inoculation of suckling mice has become a standard method to generate dengue serotype-specific antigen (for description of technique see section on HA and HI assays). For routine viral isolation however, the technique is slow and cumbersome and requires a steady supply of suckling mice. For these reasons IC of suckling mice has been supplanted with more reliable and sensitive methods discussed below.

Mosquito inoculation

Mosquito inoculation of patient sera as a technique to isolate DENV was developed in the 1970's taking advantage of the natural vector of DENV as a biological amplification system (Rosen and Gubler, 1974). A variety of mosquito vectors have been used including *Toxorhynchites splendens*, *Aedes albopictus*, or *Aedes aegypti* (Gubler *et al,* 1979; Rosen and Shroyer, 1985; Rosen *et al,* 1985). *Toxorhynchites splendens* mosquitoes have the advantage of being larger and easier to inoculate with human sera (Rosen and Shroyer, 1985). There is no risk of spreading the virus through these mosquitoes, as *Toxorhynchites splendens* are non-blood feeders.

At the AFRIMS laboratory, *Toxorhynchites splendens* is used by injecting 0.02 µl of human sera intrathoracically into the mosquito. After an incubation period of 10-to-14 days, DENV is detected in the mosquito head using anti-DENV immunofluorescence antibody. If positive, the mosquito body is triturated and inoculated onto C6/36 cells for virus expansion and typing. Isolation rates using this method are higher than direct inoculation onto cell lines with isolation rates nearly equivalent to molecular detection of virus

by RT-PCR (Vaughn *et al,* 2000). The requirement for insectaries restricts the use of this approach.

Cell culture inoculation

A variety of cell lines are permissive for DENV infection and can generally be divided into insect or mammalian cell lines. Insect cell lines in common use for propagating DENV are the mosquito derived cell lines: AP-61 from *Ae. pseudoscutellaris*, C6/36 from *Ae. albopictus* and TRA-284 from *Tx. amboinensis* (Varma *et al,* 1974; Igarashi, 1978; Kuno, 1982). Of the three, C6/36 is the most commonly used due to its ease of use, availability, and minimal background when using a dengue typing EIA or direct fluorescent antibody staining.

Mammalian cells in common use are derived from hamsters, BHK-21, or primates such as LLC-MK2 or Vero. Mammalian cells offer the advantage of cytopathic changes and plaque formation when infected (Yuill *et al,* 1968). Therefore, mammalian cells are commonly used in the plaque reduction neutralization assay. For viral isolation, mosquito cell lines are more sensitive than mammalian cells (Rosen and Gubler, 1974). Isolating virus in insect cells and plaque quantifying a second specimen on mammalian cells can combine use of insect and mammalian cell types.

One approach to quantify DENV by plaque formation on monolayers of mammalian cells in culture is to dilute samples containing virus serially ten-fold and then to inoculate 0.2 ml of each dilution onto duplicate wells of cell monolayers in 6-well plates. After a 1-hour virus adsorption, cells are overlayed with agarose in maintenance medium. After an appropriate incubation period, usually five to six days, plaques are detected by staining the monolayers with neutral red, which stains living cells. Therefore, plaques are visualized as clear areas in a red background. The virus titer, reported as PFU per 0.2 ml is calculated as the average number of virus plaques counted at a given dilution (containing about 30-100 PFU) multiplied by the dilution factor (Putnak *et al,* 1996).

Dengue virus serotype identification

Methods to identify the infecting DENV serotype in the serum of a dengue patient based on the antibody response are limited due to the high degree of antibody cross-reactivity among the DENV serotypes (Kuno *et al,* 1993). While molecular approaches using RT-PCR have evolved significantly in recent years, the standard for DENV serotype identification remains the isolation of the virus by animal, cell culture or mosquito inoculation, further expansion in cell culture, and serotype identification using DENV serotype-specific monoclonal antibodies in an immunofluorescence assay or an antigen capture EIA format (Henchal *et al,* 1983). Common monoclonal antibodies used for serotype identification include 1 F1 for DENV-1, 3H5 for DENV-2, 5D4 for DENV-3 and 1 H 10 for DENV-4 (Kuno *et al,* 1987; Malergue and Chungue, 1995).

Antigen capture EIA for the identification of DENV has been demonstrated to be a simple and reliable technique (Kuno *et al,* 1985). At the AFRIMS laboratory, an antigen capture EIA utilizes virus isolated in *Toxorhynchites splendens* mosquitoes after expansion using C6/36 cells. Immulon U plates are sensitized with goat anti-mouse IgG in each well. Dengue serotype specific mouse monoclonal antibodies (4G2 anti-flavivirus, 1 F1 anti-DENV-1, 3H5 anti-DENV-2, 10C10 anti-DENV-3, 1H10 anti-DENV-4, and 2H2 anti-dengue group) are bound onto the plate followed by capture of the unknown DENV serotype. A colorimetric reaction is formed after the addition of anti-flavivirus-horse-radish-peroxidase and its substrate. DENV serotype specific mouse brained derived antigen (DENV-1 Hawaii, DENV-2 NGC, DENV-3 H87, DENV-4 914669) are used in parallel as positive controls. Optical density (OD) is read at a wavelength of 492 nm and the results are interpreted by comparing with positive and negative controls where positive control OD is always ≥0.20 and a negative control OD is <0.20. Matching the highest OD reading to the positive dengue control serotype identifies the DENV serotype (Kuno *et al,* 1985).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is the staining of tissue specimens for the presence of specific

proteins. Dengue antigen staining is a powerful technique to diagnose dengue in fatal cases when serology is non-diagnostic and virus isolation is not available or not successful. A number of techniques have been used to detect dengue antigen in tissue specimens to include both direct and indirect fluorescent antibody staining, and enzyme conjugates using peroxidase and phosphatase conjugates (Boonpucknavig *et al,* 1975; Boonpucknavig *et al,* 1976; Boonpucknavig *et al,* 1981; Hall *et al,* 1991).

At the AFRIMS laboratory, tissue specimens are fixed in Millonig's formalin for 2 hours, irradiated in a microwave oven and then embedded in paraffin. Viral antigen is detected by performing a modification of the immunoalkaline phosphatase method as described by Hall *et al* (1991). The staining method of the tissue section involves deparaffinization in absolute alcohol and water, immersion in a 0.05% solution of protease VIII, application of HistoMark Blue® (Kirkegaard and Perry Laboratories: Gaithersburg, MD), and blocking with normal horse serum and bovine serum albumin. The specimen is incubated overnight with polyclonal mouse dengue antibody followed by a secondary biotinylated horse anti-mouse IgG then streptavidin-alkaline phosphatase, AS-B1 phosphate, hexazotized new fuchsin and levamisol, as the chromogenic substrate. The tissue specimen is then counterstained with Mayer's hematoxylin.

GENOME BASED ASSAYS

Hybridization probes and polymerase chain reaction (PCR)

In 1987, Henchal and colleagues described molecular techniques to diagnose acute dengue using slot-blot nucleic acid hybridization with a radiolabeled eDNA probe (Henchal *et al,* 1987). In 1991, reverse transcription (RT) of viral RNA followed by polymerase chain reaction (PCR) allowed the rapid (less than 12 hours) detection of DENV in patient sera. A nested technique allowed for the serotype specific diagnosis of dengue (Henchal *et al,* 1991; Morita *et al,* 1991; Lanciotti *et al,* 1992).

Henchal's slot-blot nucleic acid hybridization technique used a radiolabeled eDNA probe to detect as little as 11 plaque forming units of each of the four DENV serotypes (Henchal *et al,* 1987). Unlike antibody based assays that rely on the appearance of dengue-specific IgM or IgG, molecular techniques offer the advantage of detecting the virus directly, early in the course of a DENV infection. Nucleic acid hybridization is not affected by antibody and the appearance of virus-specified RNA coincided with the detection of antigen in infected cells.

A modification of this technique by Ruiz and colleagues utilized a microplate hybridization method (Ruiz *et al,* 1995). DENV RNA was isolated from serum or tissue samples and immobilized onto wells followed by hybridization with a biotin-labeled eDNA-probe with signal detection by peroxidase conjugation. This assay was found to have a sensitivity of 95% and specificity of 100% for all four DENV serotypes.

Henchal and colleagues developed a universal set of sense and anti-sense oligomeric DNA primers that matched all known DENV sequences (Henchal *et al,* 1991). This RT-PCR was found to be 80% sensitive and 100% specific for acute dengue compared to virus isolation in live mosquitoes. Modifications to this assay by Lanciotti *et al* (1992), using nested RT-PCR techniques have increased sensitivity and reduced assay time to less than 6 hours.

The dengue RT-PCR assay provides a rapid, sensitive, diagnostic tool to detect DENV in patient specimens as well as in the mosquito vector (Chan *et al,* 1994). The primary limitation for patient diagnosis is that DENV viremia occurs early in the course of infection and drops to non-diagnostic levels soon after defervescence (Vaughn *et al,* 2000). Other limitations include the need for a laboratory equipped with a ultra-centrifuge, thermocycler, and electrophoresis equipment. The use of positive and negative controls is essential and strict adherence to specified techniques are required to eliminate cross-contamination with RNA and DNA to produce false positive results. Despite these

limitations, dengue RT-PCR is a powerful tool to diagnose dengue serotype-specific viremia. Newer RT-PCR techniques are being developed that may be more practical for the developing dengue diagnostic laboratory including pocket thermocyclers with gel cartridges containing all the essential reagents that can be used in the field and require minimal technical expertise.

Nucleic acid sequence-based amplification (NASBA)

NASBA is an isothermal RNA amplification method that uses electrochemiluminescence to detect mRNA utilizing the NuclisensTM basic kit and the Nuclisens Reader (Organon Teknika). Unlike RTPCR, which relies on the conversion of RNA into eDNA and then amplification, NASBA directly amplifies RNA using primers and capture probes at isothermal temperatures. NASBA has been successfully used in other pathogens such as malaria, cytomegalovirus and human immunodeficiency virus (Berndt *et al,* 2000; Blok *et al,* 2000; Schoone *et al,* 2000; Witt *et al,* 2000). Recently, NASBA has been applied to the diagnosis of dengue (Wu *et al,* 2001). Using spiked sera, NASBA had a detection threshold of 1 to 10 PFU/ml. When tested against clinical samples, a threshold of 25 PFU/ml was observed, a 100% serotype concordance with viral isolation, and a sensitivity of 98.3% and specificity of 100%. NASBA though preliminary in results, may prove to be a useful diagnostic tool in the early viremic phase of acute DENV infection.

Fluorogenic probe-based 5′ exonuclease assay (Taqman)

The fluorogenic probe-based 5′ exonuclease assay (Taqman) using the PerkinElmer Applied Biosystems automated sequence detection system 7700 has been successfully used to diagnose and quantify a number of human pathogens including many viruses (Morris *et al,* 1996; Hawrami and Breuer, 1999; Jordens *et al,* 2000; Lanciotti *et al,* 2000; Loeb *et al,* 2000; Schutten *et al,* 2000). This technique is based on the use of a fluorescent-tagged probe that hybridizes with the target eDNA sequence (following the RT step). A fluorescent signal is released through the 5′-3′ exonuclease activity of DNA Taq polymerase (Holland *et al,* 1991). This allows real-time monitoring of the targeted PCR product and, with an internal control, a quantitative measurement. Taqman has been successfully used to detect and quantify DENV infection (Laue *et al,* 1999; Callahan *et al,* 2001; Houng *et al,* 2001; Warrilow *et al,* 2002). Taqman may prove to be a useful technique to rapidly diagnosis DENV infection and in particular to rapidly quantify viremia and its correlate in dengue disease severity.

Detection of dengue virus NS1 antigen using enzyme immunoassay

Alcon *et al* (2002) reported that the NS1 antigen was found circulating from the first day after the onset of fever up to day 9: NS1 levels ranged from 0.04 to 2 µg/ml in acute-phase serum samples (from days 0-to-7), and the level for a convalescent phase serum (day 8 and later) was 0.04 µg/ml. In secondary infection, the NS1 level ranged from 0.01 to 2 µg/ml and was not detectable in convalescent-phase sera (Alcon *et al,* 2002). Shu *et al* (2002) reported data from acute-phase sera with either primary or secondary infection that were in agreement with those of Alcon *et al* (2002). Moreover, their data suggested that the NS1 antigen was detectable during days 1-to-8 of illness (Shu *et al,* 2002).

Dengue genotypes studies

Although PCR is the most sensitive and rapid method for the detection of dengue virus in early stage of disease, classical dengue virus culture has important benefits: Longitudinal collections of dengue virus provide material for many studies, such as studies of pathogenesis, phylogenic characterization, as well as antigenic drift. With further sequencing using dengue serotypes seed cultures one is able to tell the genotypes in each serotype including from different years. Studies that attempt to associate virulence with genotypes clearly play an important role in the selection of parent strains for attenuated vaccines.

Dengue1-4 genotypes (Klungthong, Chonticha, Molecular section, Virology Department, AFRIMS, personnel communication) (Figs 3-6):

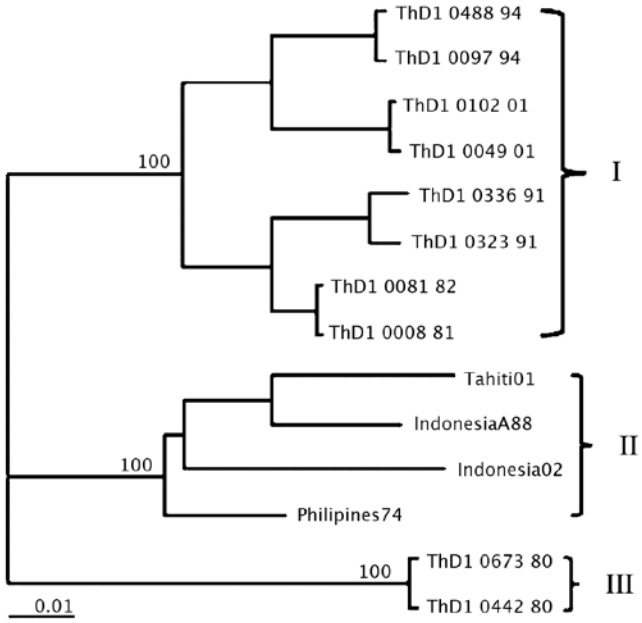

Fig 3–DENV-1 in Thailand.

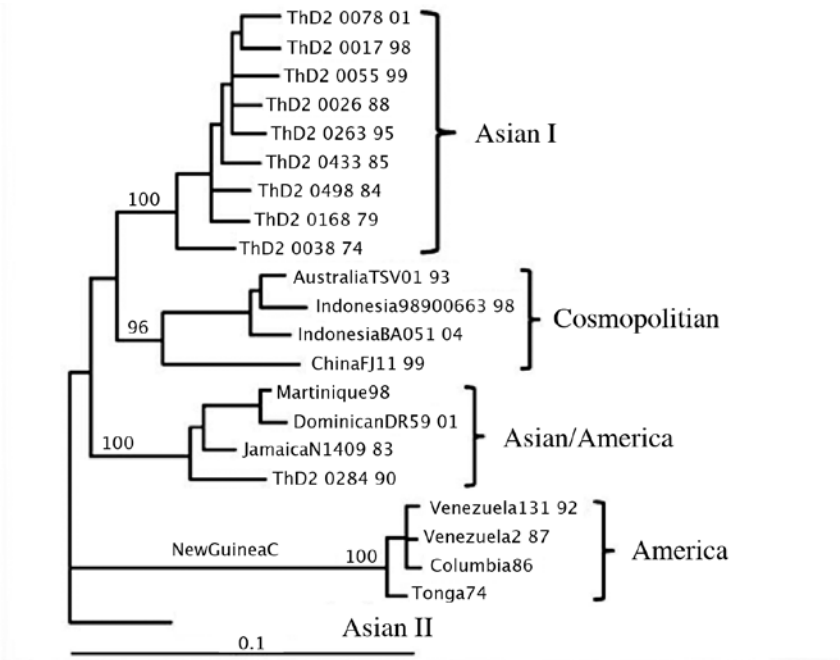

Fig 4–DENV-2 in Thailand.

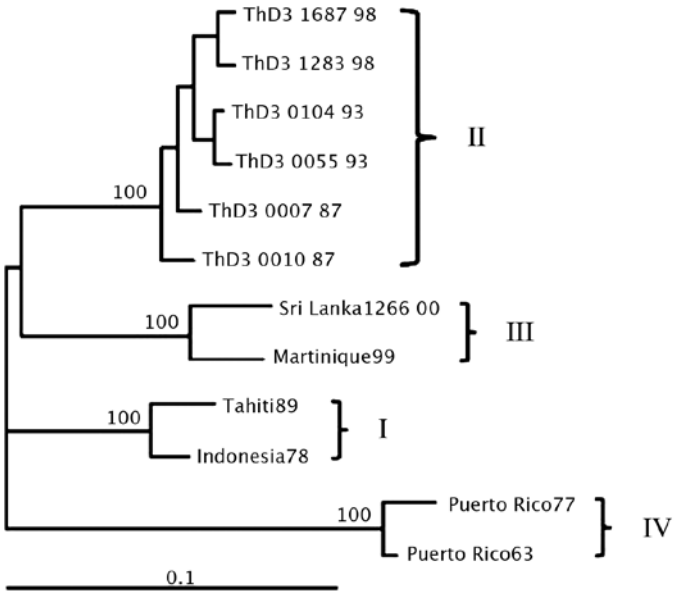

Fig 5–DENV-3 in Thailand.

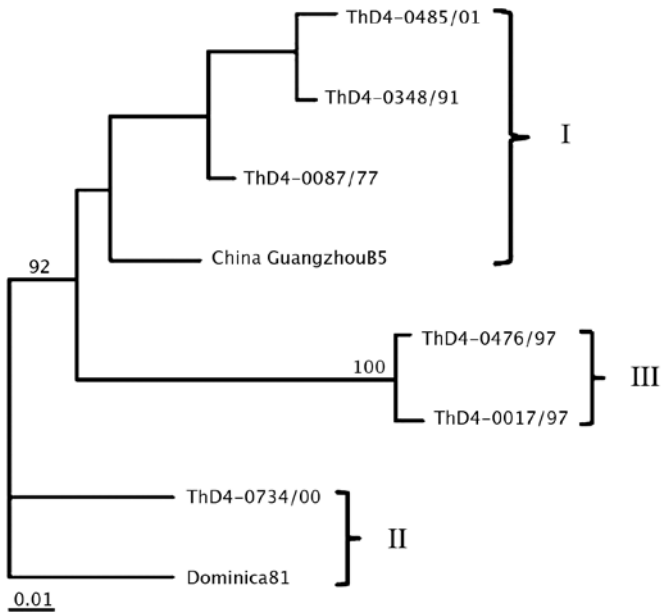

Fig 6–DENV-4 in Thailand.

DENV-1 in Thailand: Most of the DENV serotype 1 in Thailand is genotype I, circulating since 1980 to the present. A few DENV serotype 1 specimens of genotype III were found during 1980-1983, and then disappeared from circulation.

DENV-2 in Thailand: Most of the DENV serotype 2 in Thailand are Asian I genotype, circulating since 1991 to the present time. A few DENV serotype 2 specimens of Asian/American genotype were found during the 1980 to1990 period and then disappeared from circulation.

DENV-3 in Thailand: Most of the DENV serotype 3 circulating since 1990 to the present are genotype II. Genotype III was also found during 2009 to2010 (This genotype is being investigation until present).

DENV-4 in Thailand: Most of the DENV serotype 4 in Thailand are genotype I, circulating since 1998 to the present. One genotype II was found in the year 2000. A few genotype III specimens were found during the 1997 to2001 timeframe and then disappeared from circulation.

CONCLUSION

Understanding the pattern of immune responses to first or subsequent DENV infections in the context of the clinical illness is essential to identify the appropriate diagnostic tools to diagnose acute DENV infection. Antibody-based assays will not be positive early in the course of disease; patients with suspected dengue should not be sent home believing the fever is not due to dengue. They must be warned about the signs of plasma leakage and appropriately followed. Serotype specific diagnosis is difficult post-defervescence and the limitations of the antibody response in a patient with a previous DENV infection must be taken into account. With these concepts in mind, what would be the ideal assay to diagnose DENV infection? First, the assay must be both sensitive and specific, with a high predictive value despite a low incidence of disease. This will be important in countries where dengue is an emerging disease. It must have low cross-reactivity with other co-circulating flaviviruses such as JE, yellow fever or West Nile virus. The assay must be reproducible with low inter and intra-assay variability and it must be inexpensive so that developing countries with dengue epidemics might be able to utilize the considered diagnostic technique. Likewise the assay must be simple to perform with minimal training and diagnostic equipment. Such an assay would identify serotype-specific dengue antigen during the viremic period and IgM and IgG during the late acute or early convalescent period.

Given these criteria, one can visualize a rapid diagnostic test that will take minutes to perform using a finger-prick of whole blood or saliva placed onto a card with one space for a dengue serotype-specific antigen capture and another for the detection of dengue specific IgM and IgG during the late acute or convalescent period. Future assay will go beyond confirming or refuting dengue as the etiology to distinguish multiple etiologies of fever. Etiologic diagnosis based on gene expression in response to infection needs to be evaluated. These challenges for flavivirologists and commercial companies present an opportunity to aid in the control and treatment of this global health problem.

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