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1.1 Dengue and its current epidemics

The origin of word 'Dengue' is based on a Swahili phrase 'ki dinga pepo', which means a disease characterized by a sudden cramp like attack, caused by an evil spirit. Dengue is caused by dengue viruses of four closely related yet antigenically distinct serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) of the genus *Flavivirus*. Dengue is primarily affecting tropical and subtropical regions around the world, predominantly in an urban and semi urban areas. The transmission occurs through the bite of infected *Aedes* mosquitoes, principally *A. aegypti*. Dengue virus infections, range from a mild undifferentiated fever, to classical dengue fever (DF), and dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Infection induces a life-long protective immunity to that serotype but does not provide cross-protective immunity against the other three serotypes\(^1\). Therefore people who are living in a dengue hyperendemic region can have four dengue viral infections. Primary infection, infection with any one of the four serotypes, generally leads to DF. Secondary infection, infection with more than one serotype may lead to DHF or DSS.

In Southeast Asia, epidemic of DHF was first appeared in the 1950s, but by 1975 it became a primary cause of hospitalization and death among children in many countries. In the 1980s, DHF began a second expansion into Asia, when Sri Lanka, India, and the Maldives had major DHF epidemics; Pakistan first reported an epidemic of DF in 1994. The recent epidemics in Sri Lanka were associated with multiple dengue virus serotypes, but DEN-3 was predominant and was genetically distinct from the DEN-3 viruses previously isolated from infected persons in the country\(^2\). The People's Republic of China had a series of epidemics caused by all four serotypes; its first major epidemic of DHF, caused by DEN-2, was reported in Hainan Island, in 1985\(^3\). Singapore also had a resurgence of dengue/DHF from 1990 to 1994 after a successful control program had prevented significant transmission for over 20 years\(^4\). In other countries of Asia where DHF is endemic, the epidemics have become progressively larger in the last two decades. During the last 25
years, major dengue epidemics have been reported for the first time from other parts of the world such as the Kenya (1982, DEN-2), Mozambique (1985, DEN-3), Djibouti (1991-92, DEN-2), Somalia (1982, 1993, DEN-2), and Saudi Arabia (1994, DEN-2).

In India, dengue hemorrhagic fever is the main cause of hospitalization and death among children. Dengue virus was first isolated in 1945. The number of dengue cases per year ranges from 7 to 16 thousand, and all the four serotypes have been demonstrated to circulate and cause epidemics in the country. However, only occasional cases of DHF/DSS have been reported in India5. Delhi had outbreaks of dengue virus infection in 1967 (circulating virus was DEN-2), 1970 (DEN-1 and 3) and 1982 (DEN-1 and 2), but no signs of DHF/DSS were reported during these epidemics6, 7. In 1988 and 1996 (circulating virus was DEN-2) some cases of DHF were seen7. The virus was confirmed as dengue type 2, by cell culture and indirect immunofluorescence with type-specific monoclonal antibodies. In this largest out break a total of 8,900 cases were reported with 4.2% death rate.

At present, dengue is the most widespread mosquito-borne human viral disease. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific, and an estimated 2.5 billion people are living in areas at risk for epidemic transmission. The global distribution of dengue and its vector is shown in figure1.1.

In recent years there has been a rapid increase in the number of the dengue cases. Approximately 50 million cases
of DF and more than 250,000 cases of DHF occur annually. The case-fatality rate of DHF in most countries is ~5%. The reasons for this dramatic global emergence of dengue/DHF are due to lack of an effective mosquito control programme in most dengue-endemic countries, major global demographic changes, like uncontrolled urbanization and concurrent population growth, and increased air travel which facilitating the movement of different serotypes, and even genotypes from one region to another. There may be other additional factors, such as climatic changes and virus evolution that could influence the emergence of this disease9.

1.2 Dengue diseases syndromes

Dengue virus infections range from a mild undifferentiated fever, to classical dengue fever (DF), and dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS)10, 11, 12. Primary infection, generally leads to DF and secondary infection may lead to DHF or DSS.

1.2.1 Dengue fever

Infection with each of four serotypes (1, 2, 3 and 4) can cause dengue fever, which may vary in severity. Dengue fever is a mild febrile non-fatal illness, and its clinical features depend on the age of the patient. It is mild in infants and young children and severe in older children and adults. The fever with temperatures 102-105°F lasting 2-7 days11, myalgias, frontal headache, retro-orbital pain, nausea, vomiting, anorexia, altered taste and olfactory perception, and malaise, leucopenia and thrombocytopenia in some cases, were observed in the patients. In some cases bleeding complications such as gingival bleeding, gastrointestinal bleeding, haematuria, and menorrhagia were observed13.

1.2.2 Dengue hemorrhagic fever

This is the severe form of the dengue fever with additional symptoms of high fever, hemorrhagic phenomena, hepatomegaly and circulatory failure. The major physiological change in DHF from DF is leakage of plasma, as a result of an elevated haematocrit. Abdominal pain, epigastric discomfort and tenderness at right costal margin are common. Positive tourniquet test, platelet
counts <100,000 mm\(^{-3}\) and elevated haematocrit are the good indications of DHF. After 2-7 days of fever, serious stage of the disease starts with sudden fall in temperature with circulatory disturbances. At this stage patient may sweat, fatigue and some changes in pulse rate and blood pressure. Treatment for DHF patients is, to inject the fluid to counteract plasma leakage. If the patient is not treated and plasma loss is critical, leads to death\(^{13,14}\).

1.2.3 Dengue shock syndrome
This is the severe form of DHF with a rapid fall of the pulse pressure (<20mmHg), clammy skin and restlessness. Between third and seventh day of the fever, serious stage of the disease starts with sudden fall in temperature with signs of circulatory failures such as skin becomes cool, blotch, and congested, circumoral cyanosis and low pulse pressure. If proper care is not taken promptly patient may pass into a stage of profound shock, with the blood pressure or pulse becoming hardly noticeable. The duration of the shock is just 12-24 hours, before the terminal stage of the shock patients stay conscious. At this stage patients may die or recovers rapidly by appropriate volume replacement therapy\(^{15}\).

1.3 Dengue Viruses
The origin of dengue virus has been proposed that the virus may have originated from lower primates and canopy-dwelling mosquitoes in a forest cycle of Malay Peninsula. Before being adapted to lower primates and humans, the virus might have evolved as viruses of mosquitoes. The causative agent of dengue was identified in the early part of the twentieth century. After World War II, dengue became epidemic in Asian-Pacific regions\(^{16}\). Sabin was the first person to isolate dengue viruses, dengue virus type-1 (Hawaii), dengue virus type 2 (New Guinea C) in 1944\(^{17}\). Later in 1956 dengue virus type-3 and 4 were isolated\(^{18}\).

1.3.1 The structure and genome organization
Dengue viruses are classified into four groups, which share the same structure and genome organization. The virus appears as a spherical particle of 40-50nm in diameter, with a lipid envelope and membrane, enclosing a
nucleocapsid core of 30 nm in diameter is shown in figure 1.2\textsuperscript{19}. The genome of the dengue virus is ~11 Kb single-stranded RNA of positive polarity, and serves as the viral mRNA. It encodes 10 viral proteins, of which three are structural (capsid C, membrane M and envelope E) proteins, and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins is shown in figure 1.3. The genomic RNA contains a single open reading frame (ORF), flanked by 5' and 3' non-translated regions.

This ORF is translated into a single polyprotein precursor, which is cotranslationally and post-translationally processed, by both host as well as virus-encoded proteolytic enzymes, to give rise to the individual structural and non-structural proteins\textsuperscript{17, 20}. The putative roles of the dengue proteins and antibody response in humans are shown in the Table.1.

![Fig.1.2 Structure of dengue virus. A. Schematic representation of dengue virus. Abbreviations used are C: capsid; prM: premembrane; E: envelope; RNA: ribonucleic acid. B. CryoEM map of whole virus (fit of envelope dimers into density) showing envelope monomer with domains I, II and III (red, yellow and blue respectively), and the fusion peptide is shown in green.](image)

![Fig.1.3 Schematic representation of dengue virus genome. Dengue virus encodes three structural (C: capsid; prM: premembrane; E: envelope) and seven non-structural proteins (NS1, ns2a, ns2b, NS3, ns4a, ns4b and NS5). NC denotes non-coding regions.](image)
Table 1. Putative roles of dengue proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Proposed function</th>
<th>Mol. Wt. (in kDa)</th>
<th>Antibodies in patient's sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>Capsid protein, has signal sequence for M protein&lt;sup&gt;17&lt;/sup&gt;</td>
<td>12</td>
<td>Yes</td>
</tr>
<tr>
<td>PrM/M</td>
<td>Membrane protein, believed to prevent conformational changes in E&lt;sup&gt;21, 22, 23&lt;/sup&gt;</td>
<td>19/9</td>
<td>Yes</td>
</tr>
<tr>
<td>E</td>
<td>Envelope protein, host cell surface receptor, membrane fusion, major target for neutralizing antibodies&lt;sup&gt;17, 19, 24, 25&lt;/sup&gt;</td>
<td>60</td>
<td>Yes</td>
</tr>
<tr>
<td>NS1</td>
<td>Role suggested in replication&lt;sup&gt;17, 25&lt;/sup&gt;</td>
<td>48</td>
<td>Yes</td>
</tr>
<tr>
<td>NS2a</td>
<td>NS1 processing&lt;sup&gt;25&lt;/sup&gt;</td>
<td>20</td>
<td>No</td>
</tr>
<tr>
<td>NS2b</td>
<td>Required for NS3 mediated&lt;sup&gt;17&lt;/sup&gt; cleavage</td>
<td>14.5</td>
<td>No</td>
</tr>
<tr>
<td>NS3</td>
<td>Serine protease/NTPase&lt;sup&gt;25&lt;/sup&gt;</td>
<td>70</td>
<td>Yes</td>
</tr>
<tr>
<td>NS4a</td>
<td>Unknown, might be the cofactor along with the putative viral RNA dependent RNA polymerase, NS5&lt;sup&gt;25&lt;/sup&gt;</td>
<td>16</td>
<td>Yes</td>
</tr>
<tr>
<td>NS4b</td>
<td>Unknown, might be the cofactor along with the putative viral RNA dependent RNA polymerase, NS5&lt;sup&gt;25&lt;/sup&gt;</td>
<td>27</td>
<td>No</td>
</tr>
<tr>
<td>NS5</td>
<td>RNA dependent RNA polymerase&lt;sup&gt;17, 25&lt;/sup&gt;</td>
<td>105</td>
<td>Yes</td>
</tr>
</tbody>
</table>
1.3.2 The life cycle

Dengue virus gains entry to cells by either of two known mechanisms. In one case dengue virion complexes with non-neutralizing antibody, this bound antibody Fc region interacts with Fcγ receptors of the macrophage or the monocytes and this process apparently serves the entry of the virus into the cell. This is the ADE-mediated entry pathway implicated in DHF. Alternatively, virus gains entry into the cell by receptor-mediated endocytosis in which domain III of the envelope protein plays an important role.

The infectious virus enters the cell through receptor-mediated endocytosis as depicted in the figure 1.4. The low pH with in the endosomal vesicle causes conformational change and exposure of E fusogenic domain, which is important for infectivity by mediating fusion of virus to vesicle membrane thereby allowing uncoating of virus. In endoplasmic reticulum the viral genome is translated by host cell polymerases into a polyprotein precursor, which is co- or post-translationally processed jointly by host as well virus-encoded proteases.

This results in the generation of viral proteins including the presumed replicase components NS3 and NS5, paving the way for RNA replication. The (+) RNA is replicated to yield the complementary (-) strand, which in turn serves as a template for additional (+) strand synthesis. In the later stages,
the (+) RNA genome associates with C proteins to form nucleocapsid. This encapsidated genome buds into ER lumen and acquires host ER envelope. The E and prM proteins inserted into rough ER membranes during protein synthesis. Rapid assembly and maturity of virions occurs with in the intracellular vesicles. prM-E heterodimer prevents premature activation or exposure of E protein fusion domain of new virions with in vesicle. Finally, the virions are released by exocytosis via host cell secretory pathway.

1.3.3 The transmission
The principle vector for dengue transmission is female Aedes mosquito. The transmission cycle of dengue virus by the mosquito Aedes, is begins with a dengue-infected person. The person will have virus circulating in the blood that lasts for about 4-5 days\textsuperscript{30, 31}. At this stage an uninfected female Aedes mosquito bites the person and ingests blood that contains dengue virus. Then within the mosquito, the virus replicates during an extrinsic incubation period of eight to twelve days\textsuperscript{1, 32, 33}. The mosquito then bites a susceptible person and transmits the virus to that person, as well as to every other susceptible person the mosquito bites for the rest of its lifetime. Infected mosquito may also transmit the virus to the next generation of mosquitoes by transovarial transmission. The virus then replicates in the second person and produces symptoms. In the mosquito, the virus replicates within the midgut epithelium, brain and salivary glands and also replicates in the females genital track and may infect her progeny. The figure 1.5 depicts the transmission of dengue virus by the mosquito vector.

Humans provide the major amplifying reservoir for the virus and the symptoms begin to appear an average of four to seven days after the mosquito bite, this is the intrinsic incubation period, within humans. While the intrinsic incubation period averages from four to seven days, it can range from three to 14 days. The viremia begins slightly before the onset of symptoms. Symptoms caused by dengue infection may last three to 10 days, with an average of five days, after the onset of symptoms.
1.4 Present status of dengue diagnosis

Dengue infections may be clinically in apparent or may result in non-specific febrile illness, DF or DHF\textsuperscript{34, 35}. Severe plasma leakage can lead to fatal DSS and mortality rates for untreated patients can be as high as 40-50\%\textsuperscript{36}. Early diagnosis, followed by supportive care and symptomatic treatment through fluid replacement are the keys to survival in cases of severe dengue infection\textsuperscript{34, 37}. Definitive diagnosis of dengue infection depends on the identification of the virus, virus-encoded antigens, viral genomic RNA or the virus-induced antibodies\textsuperscript{38, 39}. At present there are three basic methods used for the diagnosis of dengue virus infection are virus isolation and characterization, detection of the genomic sequence by a nucleic acid amplification technology assay, and the detection of dengue specific antibodies\textsuperscript{39}. Virus identification through its isolation can take several days and may not always be successful due to very small amounts of viable virus in the clinical samples. Viral antigens can be detected by immunohistochemistry or immunofluorescence. However, the complexity of these assays and their high cost preclude their routine use. Viral RNA can be detected with a high degree of sensitivity, using coupled reverse transcription and polymerase chain reaction (RT-PCR). Besides being expensive and requiring sophisticated equipment, the RT-PCR assay is subject to wide variability, as
demonstrated in a recent study conducted by the European Network for Diagnostics of Imported Viral Disease\textsuperscript{40}. Further, a shortcoming common to all these methods is the narrow time window (\(~5\) days), available for successful detection, which coincides with the febrile period during which patients are viremic\textsuperscript{30, 31, 41, 42}. Often, dengue infected patients do not seek immediate medical care as the initial manifestations are usually asymptomatic or mild fever. This precludes diagnostic tests based on the identification of the virus or its RNA genome because of the short duration of viremia. Thus, in a majority of cases the only feasible diagnostic test would have to be based on the identification of anti-dengue antibodies.

1.4.1 Virus isolation

In the 1940s, dengue viruses were first isolated by inoculation of patient serum into suckling mouse brain. Intracerebral inoculation of 1-to-2 day-old suckling mice was the first method used to isolate the dengue viruses\textsuperscript{43, 44}. This method was found to be slow, cumbersome and currently considered the least sensitive isolation system. In 1960s, mammalian cell lines such as Vero (monkey kidney), LLC-MK2 (monkey kidney), BHK-21 (baby hamster kidney) and PS (pig origin) were popularly used\textsuperscript{43}. The sensitivities of these cell lines were generally higher than suckling mice but inferior to either mosquito inoculation or mosquito cell culture.

In the early 1970s, the mosquito inoculation technique was revolutionized the dengue virus isolation due to its higher sensitivity. The adult mosquitoes are inoculated intrathoracically with serum or tissue extracts, after 14 days, the presence of the virus is detected by using immuno fluorescent antibody test. As mosquito is the natural host, result of the virus isolation rates were of greater than 80 percent. On the other hand this method needs special insectory facilities, technical training and labor intensive. Only male mosquitoes are used for inoculation to prevent the risk of escaping female mosquitoes, and no clear signs of the disease of infected mosquitoes.

In the early 1980s, mosquito cell culture was established and it is the best system with its sensitivity, easy to handle and cost effective. It is routinely
used in many diagnostic laboratories. The cell lines, C6/36 from *Ae. Albopictus*, AP-61 from *Ae. pseudoscutellaris*, TRA-284 from *Tx. amboinensis*, have commonly used. Among these TRA-284 is the most sensitive, but C6/36 cell line is widely used because of its stability of sensitivity and ease of maintenance\(^{44}\). Currently, inoculation of the C6/36 cell line with patient's sera is the method of choice for dengue virus isolation.

The inherent disadvantages of all the above methods are (i) blood should be obtained during the febrile period or before the fifth day after the onset of illness, (ii) delivering it to the laboratory, (iii) formation of immune complex (virus-antibody) in secondary infection (large quantities of antibodies) may interfere with virus isolation\(^{43}\) and (iv) time consuming. Virus isolation by cell culture and from mosquitoes remains the standard method, although it has gradually been replaced by molecular and serological methods for rapid diagnosis\(^{39}\).

### 1.4.2 Molecular diagnosis

Molecular diagnosis has changed in recent years for reliable rapid diagnosis of dengue and other pathogens. These methods depend neither upon the growth of the virus nor the antibody development. They minimize the problems associated with virus isolation, like loss of viability of the virus due to poor storage conditions or presence of immune complex. Molecular assays can be divided into two methods, nucleic acid amplification and nucleic acid hybridization methods\(^{39,43}\).

**Nucleic acid amplification methods:**

These are reliable and sensitive. Several RT-PCR protocols are published for dengue virus detection. The first RT-PCR was originally reported by Lanciotti *et al.* in 1992\(^{45}\). In 1998 it was modified into a single step multiplex RT-PCR by Harris *et al.*\(^{46,47}\). These assays have used the core to premembrane gene regions as the target regions. These assays can detect and differentiate the four dengue serotypes by analyzing the amplified product in the agarose gel.

In 2001, Wu et al. developed the nucleic acid sequence-based assay, is an isothermal RNA-specific amplification assay to detect viral and bacterial RNA
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in clinical samples. This had a high degree of sensitivity and specificity, can be conducted at 41°C and it is isothermal. Recently real-time RT-PCR methods have reported for detection of dengue virus from serum samples. Real time PCR methods have replaced conventional PCR methods due to their rapidity, quantitative measurements, lower contamination rate, higher sensitivity, higher specificity and ease of standardization. Among real-time PCR methods TaqMan assay, is highly specific due to the sequence-specific hybridization and is most widely used.

Major disadvantages of the PCR methods are, requires technical expertise, subject to amplicon contamination and most of the PCR methods published to date did not show good sensitivity and specificity out side the developing laboratories.

Nucleic acid hybridization:
This method with dengue viral RNA was used in 1987 by Henchal et al. In this method, specifically a dot blot nucleic acid hybridization test is done using RNA extracted from dengue virus-infected cell culture supernatants with biotinylated probes or 32p-labelled probes. This method is sensitive and it is more suitable for laboratory due to its disadvantages like, the technical expertise required and it is expensive.

1.4.3 Serological diagnosis

Serological diagnosis is the commonest method to confirm dengue virus infection. These methods are easy to do, very fast, reliable and cost effective. It does not need to, isolate virus, special training and laboratory conditions. Though it is complicated by the presence of cross-reactive antigenic determinants shared by all four dengue serotypes and other flaviviruses. Serological diagnosis can be broadly divided into two assays, (i) antigen detection assay, and (ii) antibody detection assay.

1.4.3.1 Antigen detection

Progress towards the antigen detection in serum samples has been slow due to the low sensitivity of the assay for patients with secondary infection due to the preexisting virus-IgG antibody immune complexes. However recent
studies that used E/M and NS1 antigens in ELISA and dot blot assay established that high concentrations of the E/M and NS1 antigens in the form of immune complexes were detected in both primary and secondary dengue infections up to 9 days after the onset of the infection. Koraka et al. in 2003 reported that NS1 antigen in dot blot immunoassay in both nondissociated and dissociated serum or plasma samples from patients with primary and secondary dengue infections gave more number of antigen-positive patients than RT-PCR results\(^{39,49}\). These results suggest that the NS1 antigen detection can be used in early diagnosis of dengue diagnosis. However further studies are needed to evaluate and compare the sensitivities and specificities of RT-PCR and NS1 antigen detection assays\(^{39,43}\).

### 1.4.3.2 Antibody detection

Antibody detection assays are the most common rapid methods used in dengue diagnosis. Several methods have been described for the detection of dengue specific antibodies. These methods are as follows, Hemagglutination inhibition assay, Neutralization assay, ELISA, Fluorescent antibody test, Complement fixation assay and Immunoblot assay. Among these capture IgM and IgG ELISAs, antigen coated indirect IgM and IgG ELISAs and Hemagglutination inhibition assay are commonly used methods\(^ {39,43}\).

**Hemagglutination inhibition assay (HI):**

It was developed by Clarke and Casal in 1958. It is the traditional method used to detect and differentiate primary and secondary dengue infections. This method is simple, sensitive and having high reproducibility. This test is based on the ability of antibodies to inhibit viral glycoprotein dependent agglutination of goose or human ‘O’ red blood cells. If HI titer of sera is greater than or equal to 1:2,560 are classified as having secondary dengue infection, if titer is less than 1:2,560 are considered as having primary dengue infection. Due to its inherent disadvantages like, required paired sera, time consuming and fails to discriminate between closely related flaviviruses, has gradually been replaced by capture IgM and IgG ELISA\(^ {39,43,15}\).
Plaque reduction neutralization assay (PRNT):
This is a traditional serologic technique to detect dengue infection. It is the best method to evaluate the immunity after vaccination. In this test, serially diluted heat-inactivated sera are incubated with defined amount of sera. The non-neutralized viral fraction is subsequently absorbed onto a monolayer of susceptible cells and produce plaques. The percent reduction in virus plaques (50% end point) was determined with reference to a control serum. Due to its inherent disadvantages like, time consuming, expensive, technical expertise and fails to discriminate dengue serotypes, has been confined to laboratory.

Fluorescent antibody test:
In 1973 Vathanophas et al. developed the fluorescent antibody test to detect IgM and IgG antibodies in sera. This method has been limited to the laboratory because it require experienced personnel to detect significant changes in the fluorescence endpoint and broadly cross-reactive among flaviviruses.

Complement fixation assay:
Complement fixation test was developed by Casey in 1965. It is the least sensitive serological assay and difficult to perform.

Enzyme linked immunosorbent assay (ELISA):
The advantages of ELISA tests are, (i) more sensitive, (ii) convenience, (iii) many sera can be tested in a single day, (iv) no serum processing is required and (v) few microliters of sample is sufficient for a test. Among ELISA tests, the E/M-specific capture IgM and IgG ELISA is the most powerful assay for dengue diagnosis due to its simplicity, sensitivity and specificity. Many diagnostic kits are in the market with variable sensitivities and specificities. Dengue IgM/ IgG antibody detection ELISA is rapid and can detect large number of samples at a time. The limitation of this test is, in primary infection anti-dengue virus IgM antibodies start developing after 3 to 5 days of illness and decline undetectable levels over 2 to 3 months. Anti-dengue virus IgG appears after second week of illness. In secondary infection both IgG and IgM appear earlier, high titers of IgG antibodies appear before or
simultaneously with the IgM response. The disadvantages associated with most ELISA based kits are, lower sensitivity and cross reactivity with other flaviviruses, because most of the ELISA kits use virus lysate (DEN-1, 2, 3 and 4) as an antigen\textsuperscript{39, 43, 39, 57, 58, 59, 60}.

\textit{Immunoblot assays and immunochromatography tests:}

These tests are rapid and have the potential to replace more burdensome serological tests like HI and ELISA tests. These tests are best screening tests for clinicians in the hospitals\textsuperscript{61, 62, 63, 64}. Most of these kits can simultaneously detect dengue IgG and IgM antibodies in serum, plasma or whole blood with in 5 to 30 min. Some kits can differentiate primary and secondary dengue infections\textsuperscript{39, 43}. These kits have high sensitivity for IgG detection but lower sensitivities for IgM detection. The disadvantage of these tests is that they need correct visual judgment of color intensity. These kits should not be used in public health settings or seroepidemiological studies due to the high sensitivity of these assays for detection of IgG and the long persistence of cross-reactive flavivirus IgG antibodies in dengue endemic areas\textsuperscript{65, 66, 67, 68, 69}.

\textbf{1.4.4 Currently available dengue diagnostic kits}

Numerous dengue diagnostic kits (Table.2), in a multiplicity of formats, have become available recently\textsuperscript{70, 71}. Most of these kits (PanBio\textsuperscript{72}, Diagnostic Automation Inc\textsuperscript{73}, NovaTec\textsuperscript{74}, Bio-Quant\textsuperscript{75}, rely on the use of whole virus antigens, (produced in tissue culture or suckling mice brain), for the detection of anti-dengue antibodies in patient sera, and are consequently associated with an inherent biohazard risk. One kit, which has replaced the whole virus antigen with insect cell-expressed dengue envelope protein, eliminates this risk\textsuperscript{76}. However, all these kits are expensive due to the high costs associated with antigen production, making them unaffordable for use in the economically weaker countries where dengue is mostly prevalent. Apart from this, a major shortcoming of the commercial kits is that they do not differentiate between infections due to the dengue and other flaviviruses (such as Japanese
encephalitis and yellow fever viruses). Additionally, sera from patients with typhoid, malaria and leptospirosis also tend to score positive using these kits.

Table 2. Commercially available dengue diagnostic kits

<table>
<thead>
<tr>
<th>Company</th>
<th>Country</th>
<th>Antigen</th>
<th>IgG/IgM</th>
<th>Cost</th>
<th>Specificity</th>
<th>Sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomedicals</td>
<td>Germany</td>
<td>Total virus, Envelope</td>
<td>Both</td>
<td>High</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Pathozyme</td>
<td>Germany</td>
<td>Virus</td>
<td>IgM</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Cypress</td>
<td>Belgium</td>
<td>Virus, (D-2)</td>
<td>Both</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>GV</td>
<td>India</td>
<td>Purified Virus</td>
<td>Both</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Biomedical Automation, Inc.</td>
<td>USA</td>
<td>Purified Virus</td>
<td>Both</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

1.5 Dengue diagnostic epitopes

1.5.1 Methods for epitope identification

1.5.1.1 Computer assisted prediction

Antigenic regions or immunodominant epitopes are predicted by using (i) hydrophilicity, flexibility, surface probability, accessibility profiles and antigenic index (ii) regions predicted to form surface loops according to prediction of secondary structure, (iii) regions with high sequence variability, containing insertions or deletion sites comparing sequences of dengue and other flaviviruses and (iv) regions containing glycosylation sites in dengue and other flaviviruses (Figure 1.6) 81, 82, 83.
Figure 1.6. Computer prediction of dengue virus antigenic regions. (A) Antigenic index, (B) Kyte and Doolittle hydrophilicity and (C) transmembrane regions of complete amino acid sequence of DEN-2 virus was analysed by Mac software programme.

1.5.1.2 Phage display

It is a powerful technique that allows the discovery and characterization of proteins that interact with the desired target. Phage display is a system in which a protein or peptide is displayed on the surface of a phage as a fusion with one of the coat protein of the virus. By cloning large number of DNA sequences into the phage, display libraries are produced with a range of many billions of unique displayed proteins or peptides (Figure 1.7) 84, 85, 86.
Using a process of biopanning, one can rescue phages that display a protein or peptide that specifically binds to the target or antibody. In brief, a simple method for biopanning involves coating a plate with the target and incubating the library on the plate to allow a phage displaying a complementary protein or peptide to the target to bind. Phage, which did not bind are then washed away and those that are bound are eluted. Infection of bacteria with the binding phage results in phage amplification. Successive rounds of biopanning enrich the pool of phage with clones specifically bind the target. DNA sequencing of the phage genome determines the amino acid sequence of the protein or peptide binding to the antibody.

1.5.1.3 Peptide scanning

The identification of regions of interaction between an antigen and antibody is an important area of research in molecular immunology. The correct identification of epitopes not only allows one to map where the important regions of an antigen are located in its three-dimensional structure but more importantly, it is instrumental in the diagnosis, in the design of drugs, generation of vaccines where specific peptides are used to induce antibodies to pathogenic organisms. Various methods have been used to identify epitopes, these include predictive algorithms, which identify possible epitopes, and these are then synthesized and screened. One of the prerequisites to use algorithms for epitope prediction is that the amino acid sequence of the
protein needs to be known and the major drawback is that the predictions are not always accurate. Enzymatic and chemical cleavage using cyanogen bromide have also been used to generate peptide fragments, which are then screened to locate epitopes on the fragments. Once the fragment is identified, it is sequenced and the epitope characterized. The amino acid sequence of the protein is not required initially for this method. A popular method for identifying epitopes is epitope mapping using synthetic overlapping peptides spanning the entire sequence of the protein of interest. The synthesis of peptides can be carried out using resin-based technologies where the peptide is synthesized on the resin and then cleaved. Using this conventional system of protein synthesis, milligram amounts of protein is produced but only one peptide can be synthesized at a time. The bottleneck in epitope analysis therefore arose at the peptide synthesis level. For an epitope mapping method the two considerations of importance were that the method would allow the parallel synthesis of a large number of peptides and subsequent testing of large numbers of samples. The ability to identify epitopes speeded up enormously by a novel development in solid phase peptide synthesis and testing known as Pepscan technology developed by Geysen et al. The Pepscan method utilizes solid phase synthesis of peptides on polystyrene pins. In this method, multiple peptides are synthesized essentially by Merrifield's solid phase protein synthesis method on specially designed polystyrene pins in a 96-well microtitre plate format which are then screened against sera or antibodies of interest using ELISA to identify linear B cell epitopes. This
novel development of solid phase peptide synthesis on plastic pins as the support for the synthesis in a 96-well format has permitted easy and efficient synthesis and subsequent screening of large numbers of peptides by ELISA. Which allows the simultaneous synthesis of hundreds to thousands of peptides. Peptides are synthesized on the surface of the derivatised polyethylene or polypropylene components (Figure 1.8). Major advantage of the technique is that peptides are covalently bonded with the surface of the pins. So that the peptides can be reused in ELISA after removing the bound antibodies by harsh procedures.

Pin derivatisation:
To generate reactive surface for coupling, polyethylene gears are radiation grafted. The activated gears consists of hydroxyl groups were then esterified with Fmoc-glycine and then acetylated to cap any un-reacted hydroxyl groups to prevent them from taking any further part in peptide synthesis. Next the Fmoc protecting group is removed and free amine is coupled to Fmoc-alanine, and then acetylated. Now the pin surface is ready to couple the amino acids.\(^9\)

Peptide synthesis:
Amino acids used in the multipin peptide synthesis have their α-amino group protected with 9-fluorenylmethyloxycarbonyl (Fmoc) group and side chains are protected by the following group: t-butyl ether for serine, threonine and tyrosine; t-butyl ester for aspartic acid and glutamic acid; t-butoxycarbonyl (Boc) for lysine, histidine and tryptophan; 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; and trityl for cysteine. The peptide synthesis steps are shown in Figure 1.9.

To scan a protein of its immunodominant epitopes, the amino acid sequence of the protein is required. The amino acid sequence of the protein is determined mostly through the DNA sequence. Linear overlapping peptides of a given length spanning the entire amino acid sequence of the protein are synthesized on pins, which are attached to a plastic support (Figure 1.10). The pins are incubated with sera or antibodies of interest. The pins are then incubated with species-specific secondary antibody conjugated with enzyme.
The complex is visualized using an appropriate substrate and the color developed read in an ELISA reader. Pepscan has the potential therefore to identify linear epitopes on an antigen.

**Fig.1.9 Diagram of solid phase peptide synthesis**, showing the attachment, deprotection, coupling, repeated deprotection, and coupling steps in peptide synthesis.
Target antigen

\[
\text{RAGTSNDFPLKCVMEQHLIPGFTEDS}
\]

Synthesized scanning peptides

Fig. 1.10. Schematic representation of peptide scanning, showing the target antigen amino acid sequence (shown in different colors) on top, and below are the overlapping peptides to be synthesized.

1.5.2 Diagnostic epitopes of dengue viral proteins

Dengue viruses encode and express three structural (capsid, C; premembrane prM and envelope E) and seven non-structural (NS) proteins (NS1, 2a, 2b, 3, 4a, 4b and 5)\(^{29}\). Of these ten proteins, antibodies to C, prM, E, NS1 and NS3 have been detected in sera of dengue-infected patients\(^{90, 91, 92, 93, 94}\). A dengue viral protein with an electrophoretic mobility similar to that of NS4a has been observed in Western blots using dengue patient sera, suggesting that dengue infection elicits anti-NS4a antibodies as well\(^{91, 92}\).

The E-glycoprotein is the major structural component\(^{96}\) and the most immunogenic of all the dengue viral proteins, eliciting the first and longest-lasting antibodies\(^{91, 95}\). Immunodominant B cell epitopes on the E protein are well documented\(^{97, 98, 99, 100, 101}\). Amongst the non-structural proteins, NS1\(^{102, 103, 104}\) and NS3\(^{91, 105}\) are reported to elicit significant antibody responses, particularly in secondary infections\(^{106}\), with the former being more immunogenic than the latter. Epitopes on these proteins have been mapped using synthetic peptides spanning entire proteins\(^{98, 104}\) or defined regions based on computer predictions\(^{105, 107}\), phage displayed peptides\(^{108, 109}\) and recombinant fragments\(^{100, 110}\) on the basis of reactivity towards patient sera\(^{98, 103}\) or monoclonal antibodies\(^{100, 110, 104, 108, 109}\). While the epitopes on the
major antigens such as E\textsuperscript{97, 98, 99, 100, 101} and NS1\textsuperscript{102, 103, 104, 108} have been mapped extensively, not much information is available in the literature regarding the epitopes of the remaining proteins.

1.5.2.1 Envelope protein
The envelope glycoprotein has been mapped for the antigenic regions those elicits antibodies. The methods used for the mapping are, (i) expressing protein fragments and analyzing their reactivity's with polyclonal and monoclonal antibodies, (ii) using synthetic peptides spanning entire proteins or defined regions based on computer predictions and analyzing their reactivity's with polyclonal and monoclonal antibodies, (iii) phage displayed peptides on the basis of reactivity towards patient sera or monoclonal antibodies, (iv) solving the structure of tick-borne encephalitis virus E-protein, three antigenic domains (A, B and C) were identified on the envelope. The A-domain (aa 50-130 and 185-300) is a linearly discontinuous domain that is devided by C-domain (aa 130-184), contains most of the conformationally dependent virus neutralizing epitopes. B-domain (aa 300-400) requires a disulfide bond between Cys-11 and Cys-12. The A-B- and C-domains constitute the head of the E protein. A 100 amino acid long region (aa 400-500), that includes 50 amino acid (aa 450-500) hydrophobic tail that anchors the molecule in the virion envelope\textsuperscript{111}.

In 1989, Innis \textit{et al.}\textsuperscript{98} and Aaskov \textit{et al.}\textsuperscript{97} used pin bound hexa and octapeptides respectively, covering the entire envelope protein of DEN-2 virus. Both studies were found that most of the envelope protein was immunogenic. In the first study, the pin bound peptides were tested with dengue patients’ sera and found 25 domains containing linear epitopes. From this study, four dengue type-2 virus specific epitopes (aa 276-281, 461-467, 472-477 and 485-491) and one flavivirus group reactive (aa 205-214) epitope were hypothesized. The epitopes, aa 235-242, 248-255, 372-383 and 418-433 were reactive with all four dengue serotypes. In the second study, two peptides (aa 216-223 and 448-455) were recognized by sera from all DEN-2 infected rabbits. One of these (aa 448-455) was also reacted with both the DEN-2 patient’s sera. In the same study, binding site of the Mab-1B7 (this
neutralizes and enhances infection of den 2 and inhibits haemagglutination of erythrocytes by dengue viruses) was hypothesized to contain three discontinuous regions of the envelope (aa 50-57, 127-134, and 349-356). This epitope assignment was made because 1B7 was bound best to these three peptides. In this study two IgM epitopes were identified (aa 217-224 and 275-282), these recognized by IgM but not IgG in serum from individual rabbits, but both these peptides reacted with IgG from several of the den-2 infected rabbits.

In 1988, Markoff et al. synthesized 38 overlapping 15-mer peptides of E-glycoprotein excluding hydrophobic carboxyl terminus. Two of these peptides (aa 258-271 and 290-310) strongly reacted with mouse hyperimmune ascitic fluid. The peptides (aa 27-41, 34-48, 113-127, 197-210 and 297-310) were reacted above 50% of the den-4 patients sera tested and the peptides (aa 102-116 and 318-333) were reacted 50% of the patients’ sera tested.

In 1992, Trirawatanapong et al., used combination of both E.coli expression and synthetic peptides to precisely map neutralizing Mab-3H5 binding site (aa 386-397) and this peptide also elicited low-level virus-neutralizing antibody in rabbits. In 1990 and 1994, Roehrig et al. synthesized 25 peptides of length 9 to 39 amino acids covering most of the envelope of DEN-2. Eleven peptides defined four regions (aa 1-55, 79-172, 225-249 and 333-388) were elicited antiviral antibody and two peptides (aa 35-55 and 352-368) elicited low levels of virus neutralizing antibody in mice.

In 1999, Falconar et al. used set of overlapping peptides covering B-domain (301-401) of the envelope protein of den-2 and identified two epitopes (aa 349-359 and 274-283), which were reacted with DEN-2 anti-E Mabs.

In 1990, Mason et al. expressed fragments of envelope protein and found two immunogenic domains (aa 76-93 and 293-402). The later one required a disulfide bridge.

In 1992, Megret et al. prepared 16 overlapping fragments of DEN-2 envelope protein, expressed as trp-E fusion products in E. coli and analysed with
murine hyperimmune sera and anti-virus Mabs. Six antigenic domains were identified. One virus specific (aa 22-58) and other sub complex specific epitope (aa 304-332) were identified by non-neutralizing Mabs. Neutralizing Mabs defined two group-reactive epitopes present on two overlapping domains (aa 60-135 and 60-205).

1.5.2.2 Capsid and Membrane proteins
Little is known about the B-cell epitopes of the capsid and membrane proteins. In 2002, Vazquez et al. identified two peptides (aa 3-13 and 103-124) of premembrane elicited neutralizing antibodies against all four-dengue serotypes\textsuperscript{114}. In 1999, Falconar et al. used premembrane Mabs to identify epitopes on premembrane. They identified one epitope (aa 1-10) on the premembrane. In 1992, Bulich et al. synthesized octapeptides and reacted with Mabs of Capsid protein\textsuperscript{116}. They identified one epitope (aa 9-19) was reacted with Mabs, and these Mabs were cross-reactive with den-4 virus.

1.5.2.3 NS1 protein
It is major non-structural glycoprotein expressed on the surface of the virus-infected cell line. In 1988, Putnak et al. demonstrated that polyclonal antibodies elicited with virus-infected mouse brain antigen, reacted better with the NS1 amino-terminal region but NS1 polyclonal serum was more reactive with the C-terminal region\textsuperscript{117}. In 1997, Garcia et al. were synthesized five oligopeptides, which were predicted B-cell epitopes of NS1 of den-4 virus. Except one all the predicted epitopes (aa 33-49, 133-149, 330-346 and 169-179) were reacted significantly with the dengue patient’s sera tested\textsuperscript{103}. In 1999, Hung et al. synthesized 19 15-mer peptides, which were predicted B-cell epitopes by computer softwares\textsuperscript{102}. Among them one peptide found (aa 1-15) was IgM specific epitope. It reacted 45\% of the dengue patients sera tested. It elicits low levels of IgG antibodies in dengue patients. In 1995, 2001, Wu et al. and Yao et al. identified the B-cell epitope of NS1 (aa 110-117) of den-1 from phage display peptides by screening with DEN-1 Mabs\textsuperscript{108, 109}. This peptide reacted significantly with DEN-1 infected rabbits and patients sera. In 1994, Falconar et al. used 174 overlapping synthetic nanomeric
peptides covering the entire NS1 region\textsuperscript{104}. These peptides were analysed with 34 Mabs specific for DEN-2 virus NS1 and identified four epitopes (aa 25-33, 61-69, 111-121 and 299-307).

1.5.2.4 NS2a/2b, NS3, NS4a/4b and NS5 proteins
Antigenic structure of the above proteins is very little known when compared either with envelope glycoprotein or NS1 protein. In 1997, Garcia \textit{et al.} were synthesized one oligopeptide, which was predicted B-cell epitope of NS3 (aa 572-591) of DEN-4 virus. It was reacted significantly with the dengue patients' sera tested\textsuperscript{103}.

1.5 Future of dengue diagnosis
Inexpensive recombinant dengue proteins, which can differentiate primary and secondary dengue infection and can detect anti-dengue IgM and IgG antibodies with high sensitivity and specificity, will be good diagnostic candidates. These will be safe alternatives to diagnostic assays using whole virus antigens. Diagnostic kits which can detect anti-dengue antibodies in saliva will be very good alternatives to replace present diagnostic kits which use serum to detect antibodies. In addition to antibody testing, it will be ideal to have dengue antigen detection immunoassays with absolute specificity for dengue viruses, Moreover immunoassays which can easily serotype all 4 dengue viruses with great specificity will also be useful for the monitoring immune response to future dengue vaccine trials.