

Dengue Diagnosis: Challenges and Opportunities

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Abstract

An arthropod borne dengue viral infection is caused by dengue virus that gains entry into the human body via mosquito bites. Since dengue is a viral disease, there is no definitive drug or vaccine that can treat it, though research is going on around the world. As per the World Health Organization's estimation, nearly 2.5 billion people around the globe are at risk of this infection. In resource-limited settings, these risks are compounded by inadequate or absent diagnostic methods. With a rapid upsurge and reemergence of dengue fever and other deadly diseases, especially in undeveloped regions, a key point in avoiding the high mortality rate and reducing disease burden is diagnosing the disease at its initial stages with the help of robust, cheap and sustainable diagnostics. Complications of dengue can be well prevented with early detection followed by accurate diagnosis. This review focus on challenges, opportunities and future prospects associated with dengue infection diagnosis.

Keywords: Dengue diagnosis; Polymerase chain reaction; Biosensors; Challenges; Opportunities

Introduction

Dengue is a mosquito borne viral disease that has spread in all World Health Organization (WHO) regions in recent years. Dengue virus belongs to Flaviviridae family, is transmitted to humans by bite of an infected female mosquito mainly of the species *Aedes aegypti*, *Aedes albopictus* (to a lesser extent). The disease is widespread throughout the world (tropics), with local variations in risk influenced by unplanned rapid urbanization, increases in long-distance travel, temperature, rainfall, lack of sanitation and ineffective mosquito control. In 1950s, severe dengue or Dengue Hemorrhagic Fever (DHF) was first recognized during dengue epidemics in Thailand and Philippines. Today, severe dengue affects most Latin American and Asian countries and has turn out to be a leading cause of serious illness and deaths among children in these regions. There are four serotypes of the dengue virus i.e., DEN-1, DEN-2, DEN-3 and DEN-4 which are responsible for causing dengue. These serotypes are distinct, still closely related with each other. Recovery from one serotype infection provides lifetime immunity against that particular serotype. However, there is only partial and temporary cross-immunity to the other serotypes after recovery. Subsequent infections by other serotypes increase the chances of developing severe dengue.

Dengue virus is responsible for causing two types of infections, primary and secondary. Primary infection results in dengue fever (DF) which is acute febrile illness and cleared in approximately seven days by a complex immune response. However, secondary infection is more severe than primary infection and results in dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [1] which is characterized by following parameters:

- Increased vascular permeability
- Plasma leakage
- Hemorrhagic manifestations and
- Thrombocytopenia.

Both DHF and DSS can be fatal and can lead to death.

Currently, no effective vaccines or medicines are available to treat dengue virus. The vaccine needs to be tetravalent to be effective in

all four dengue virus serotypes and no efficient animal model is also available for testing in DHF or DSS. Therefore, developing a dengue vaccine is quiet challenging. However, several researchers around the world are working on quick priorities towards developing vaccines and medicines against this virus and many antiviral compounds are under testing against dengue infection to eliminate the disease. This problem is treated as a matter of urgency as failure to develop effective dengue control strategies will certainly result in a further rise in the number of infected humans.

Today, diagnosis of dengue at an early stage is the only effective strategy to control disease progression. This review focuses on current understanding of dengue virus pathogenesis, its life cycle, commonly used dengue diagnostic techniques, challenges and opportunities involved in dengue diagnosis.

Global distribution and burden of dengue

The incidence of dengue has dramatically grown around the world in recent decades. Actual numbers of cases with dengue are under-reported and many cases are misclassified. One recent estimate indicates about 390 million cases of dengue infections per year, of which 96 million evident clinically with any severity of disease [3]. Another study of dengue prevalence estimates that 3900 million people living in 128 countries are at risk of infection with dengue viruses [2].

Dengue virus was first isolated in 1942 from Japan by Hotta and Kimura through inoculating the brains of suckling mice with a serum of clinically ill dengue patients [4]. Before 1970, severe dengue epidemics

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were experienced by only nine countries. The disease is now endemic in more than 100 countries in the WHO regions including America, Africa, South-East Asia, Eastern Mediterranean, and Western Pacific. Out of these regions, America, South-East Asia and Western Pacific are most seriously affected. In year 2010, nearly 2.4 million cases of dengue were reported. The numbers of cases not only are increasing but also explosive outbreaks are occurring as the disease spreads to new areas. The threat of dengue fever outbreak now exists in Europe and in 2010, for the first time, local transmission of dengue was reported in France and Croatia. Although the global burden of the disease is unclear, the records of all dengue cases partly explain the sharp rising in the number of cases reported in recent years.

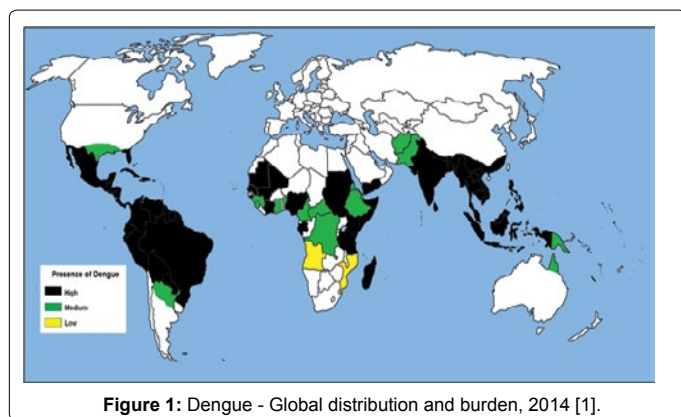
In 2012, dengue outbreak cases were reported on the Madeira Islands of Portugal and imported cases were detected in mainland Portugal and 10 other countries in Europe. As per the official data submitted by Member States to WHO, Dengue cases across the America, South-East Asia and Western Pacific has exceeded from 1.2 million in 2008 to over 3 million in 2013. Recently, the number of reported cases has increased. In year 2013, cases have occurred in Florida and China. About 2.35 million cases were reported only in America, of which 37,687 were of severe dengue. Dengue continues to affect several South American countries also, notably Costa Rica, Honduras and Mexico. In Asia, after an interval of several years, an increase in cases has reported in Singapore. In 2014, an increase in the number of cases were reported in the China, Fiji, the Cook Islands, Malaysia and Vanuatu, with Type 3 Dengue (DEN 3) affecting the Pacific Island countries after a gap of over 10 years. Dengue was also spotted in Japan after a break of over 70 years. In 2015, Brazil, several neighboring countries, Pacific island countries of Fiji, Tonga and French Polynesia are experiencing an increase in the number of dengue cases.

An estimated 500,000 people with severe dengue require hospitalization every year, out of which, a large proportion is of children. About 2.5% of those affected die. Global burden and distribution of dengue infection in year 2014 is depicted in Figure 1.

Transmission cycle of Dengue virus

As discussed earlier, dengue virus is carried and spread by *Aedes* genus of mosquitoes which include number of mosquito species. Out of these species, the primary vector of the dengue virus is *Aedes aegypti*. It is the principal vector responsible for dengue transmission and epidemics. Other mosquito species of genus *Aedes* (*Aedes albopictus*, *Aedes scutellaris*, and *Aedes polynesiensis*) serve as dengue vectors with limited ability [5].

Aedes aegypti is a small, dark mosquito. It can be identified by



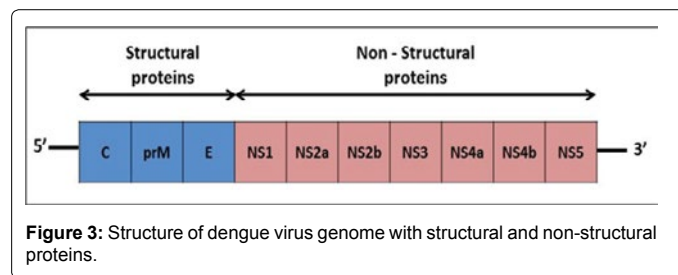
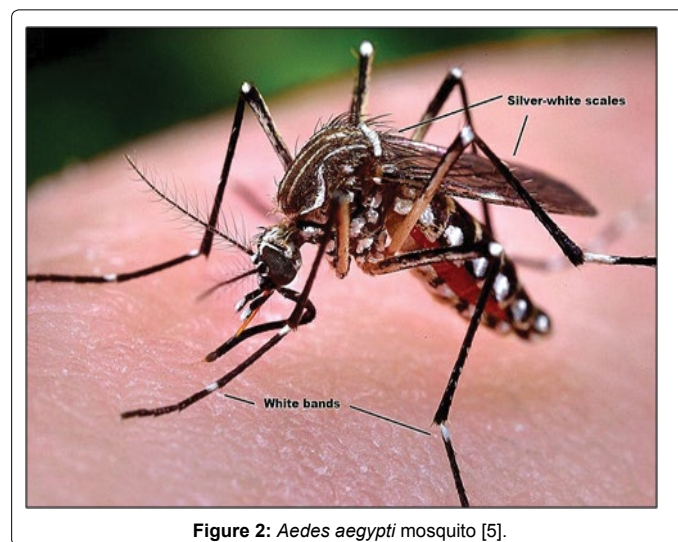
the white colored bands on its legs and a silver-white colored pattern of scales on its body (Figure 2). *Aedes aegypti* lives mainly between the latitudes of 35°N and 35°S in tropical and subtropical regions throughout the world, where the winter temperature is no colder than 10°C. Since *Aedes aegypti* require a warm climate, they usually do not live at altitudes above 1000 m, where the temperature is colder. They generally spend their entire lives in and around the houses where they hatch eggs [5].

Dengue is a single, positive stranded RNA virus belonging to family Flaviviridae and genus Flavivirus. This genus also includes Yellow fever virus, Tick borne Encephalitis virus, West Nile virus, and various other viruses. Dengue virus is 50 nm in size, and enveloped with a lipid membrane. 180 identical copies of the envelope (E) protein are attached by a short transmembrane segment to the surface of viral membrane. The virus has about 11000 genome bases encoding single large polyproteins, cleaved into several structural and non-structural mature peptides [6].

Polyprotein is basically divided into:

- Three structural proteins viz. capsid (C), membrane (prM), envelope glycoprotein (E)
- Seven non-structural proteins viz. NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5 and
- Short noncoding regions on both the 5' and 3' end (Figure 3)

The C protein binds strongly with RNA to form nucleocapsid. The structural proteins are itself divided by furine mediated cleavage from a prM which represents precursor of mature M protein. The E glycoprotein plays a role in virion attachment to the receptor, the



fusion of virus envelope with the target cell membrane and bears the virus neutralization epitopes. In addition to E glycoprotein, only one other viral protein, NS1, is associated with immunity protection. NS3 is a protease and a helicase, whereas NS5 is RNA polymerase in charge of viral RNA replication [6]. Different structural and non-structural proteins with their function are provided in Table 1.

The dengue virus is spread through a transmission cycle of human-to-mosquito-to-human (Figure 4). Typically, on fourth day after the bit of an infected *Aedes aegypti* mosquito, a person develops viremia (condition with high level of the dengue virus in the blood). Viremia lasts for minimum of five days and as long as twelve days. No sign and symptoms of dengue are seen on first day of viremia. On fifth day, the person develops symptoms of dengue fever and can last for a week or longer. After a mosquito feeds on the blood of someone infected with the dengue virus, that mosquito becomes a vector of dengue virus. This virus gets spread throughout the body of mosquitos over a period of eight to twelve days after entering into mosquito's system in the blood meal. After this period, now the infected mosquito is ready to transmit the virus into another person. Once infected with dengue virus, mosquito remains infected for its entire life and continue transmitting this virus to healthy people for the rest of their life spans, generally a three- to four-week period.

Dengue: clinical manifestations

Most dengue infections are not symptomatic. The clinical syndromes of dengue are categorized into (Figure 5):

- Undifferentiated fever
- Classic dengue fever
- Dengue hemorrhagic fever (DHF)
- Dengue shock syndrome (DSS)

Dengue shock syndrome is a severe form of Dengue hemorrhagic fever.

Undifferentiated fever

It may be the most common manifestation of Dengue. Study of Dengue infections was carried out in 4 to 16 year old students in Bangkok, Thailand which showed that about 87% students infected by dengue virus were either asymptomatic or minimally symptomatic and were found to be absent for one day in school [7].

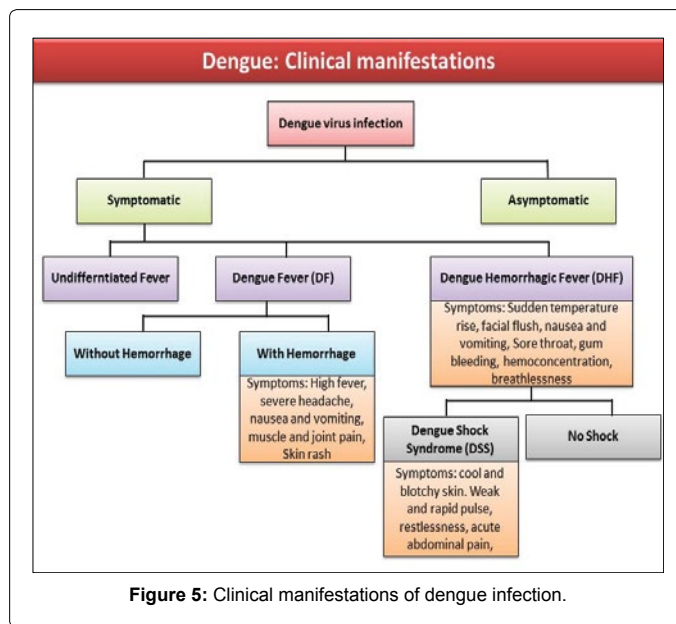
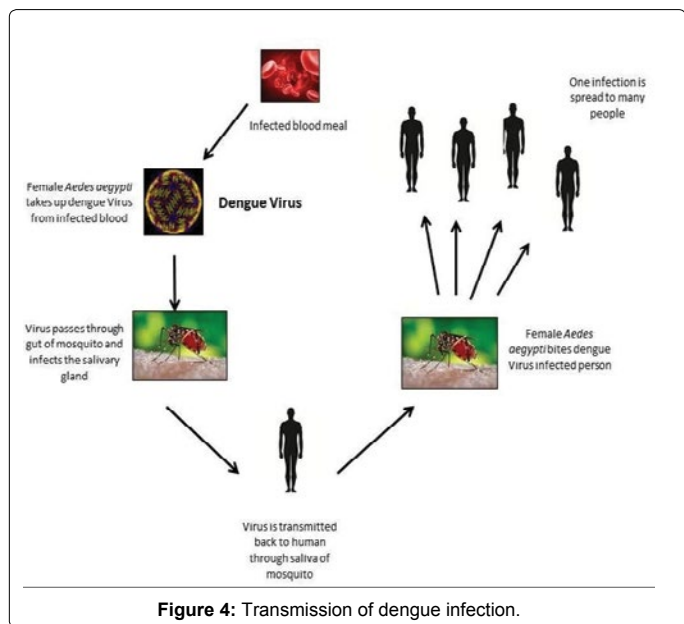
Classic dengue fever

Some of the characteristics of dengue fever are:

- Fever, with sudden onset

Protein		Molecular weight	Function
Structural Proteins	E	55-60	Cell receptor binding, envelope fusion
	prM	18-19	Proper E folding and assembly. Avoid E premature fusion during secretion
	C	9-12	Binds the RNA to form nucleocapside
Non-Structural Proteins	NS1	42-50	Viral replication
	NS2a	22	RNA synthesis and virus assembly
	NS2b	14	Cofactor of serine protease activity of NS3
	NS3	67-70	Serine protease (polyprotein processing), NTPase and Helicase (synthesis and viral RNA), and triphosphatase (capping pathway)
	NS4a	16	RNA synthesis and virus assembly
	NS4b	27	RNA synthesis and virus assembly
	NS5	104-106	RNA dependant – RNA polymerase, methyltransferase (capping pathway)

Table 1: Structural and non-structural proteins with their functions.



- Severe headache
- A rash that may present at different stages of illness with variable appearance
- Retro-orbital pain, photophobia, extreme weakness, anorexia
- Myalgias (Muscle pain) and arthralgias (Joint Pain) that can be very severe
- Nausea and vomiting
- Hemorrhagic manifestations

After 3-4 days of fever (39-40°C), temperature returns back to normal and then rises again for 2-3 days (known as saddleback fever). In some patients, dengue fever may be accompanied by bleeding such as epistaxis (bleeding from nose), gingival bleeding, gastrointestinal bleeding and menorrhagia. Some patients have also reported itching and irregularities in the sense of taste, particularly a metallic taste [7,8].

Dengue hemorrhagic fever (DHF)

There are four criteria which need to be fulfilled to meet the definition of dengue hemorrhagic fever (DHF), according to WHO. These criteria are as follows:

- Fever, or recent history of acute fever
- Hemorrhagic manifestations (bleeding including positive tourniquet test)
- Thrombocytopenia (Low platelet count - 100,000/mm³ or less)
- Hemoconcentration (a rise in hematocrit \geq 20% or signs of plasma leakage such as pleural effusion, ascites, proteinemia) [7,8]

Plasma leakage is a critical difference between dengue hemorrhagic fever (DHF) and dengue fever meaning that patient requires fluids, occasionally high amounts of intravenous fluids.

There are four grades of DHF:

Grade 1: Fever and non-specific symptoms are present and the only hemorrhagic manifestation is provoked, which is, a positive tourniquet test.

Grade 2: Along with Grade 1 manifestations, there is spontaneous bleeding.

Grade 3: Incipient shock with signs of circulatory failure.

Grade 4: The patient has profound shock, with undetectable blood pressure and pulse [7,8].

Dengue shock syndrome (DSS)

Grades 3 and 4 of Dengue hemorrhagic fever are considered as Dengue shock syndrome. Most DHF patients do not go into shock. Observations have shown that many patients with DHF who progress to shock have certain danger signs before manifesting circulatory failure. These signs include, sudden change from fever to hypothermia, with sweating and prostration, persistent vomiting, intense and sustained abdominal pain and change in mental status of the patient. For proper management and treatment of this life threatening disease, diagnostic test needs to be conducted to confirm the diagnosis of disease.

Dengue diagnosis

When dengue virus invades the human body, immune system (main defense mechanism) comprising of an innate arm responds

immediately while an adaptive arm specifically and efficiently targets the virus. An innate arm does not provide long term protection whereas the adaptive arm made up of antibodies is said to provide lifelong immunity. In the early stages, before the onset of antibodies, diagnosis usually depends on detection or isolation of the virus or viral antigens. Diagnosis of dengue is important not only for clinical management of patients, but also for intervention during outbreaks, epidemiological surveillance and for effective development of vaccine and its monitoring. Laboratory confirmation has become vital part of diagnosing dengue. The main barrier in developing an ideal diagnostic assay or technique lies in the incompletely understood pathogenesis of dengue and also in multiple sequential infections occurring in dengue endemic areas. When a person is infected with dengue virus, he or she develops full immunity towards the particular infecting serotype and not towards other three serotypes.

Around the world continuous and intensive research is being carried out, but dengue pathogenesis still remains controversial and a mystery which needs to be solved. Most of the theories such as antibody dependent enhancement, original antigenic sin, and cross reactive cellular responses focus on secondary infections than that of the first [9]. Still primary and secondary infection plays an important role in dengue diagnosis. For a diagnostic assay to be useful and effective, it is very essential for users to have confidence in the test in order to improve disease management, especially during acute early stage and for detecting severity signs. In absence of effective vaccine and antiviral therapies for dengue, diagnosis at an early stage could prove to be an important tool for timely etiological investigation, clinical intervention, and for disease control. With the possible introduction of effective vaccine in the near future, dengue diagnosis is expected to become even more important, as data from vaccine efficacy trials could determine the usefulness of candidate vaccines [10]. Diagnosis is also important for confirmation of DF or DHF or DSS, to differentiate dengue from other diseases such as rubella, leptospirosis, other flavivirus infections, and for the clinical management and evaluation of patients with severe disease [11,12].

Methods used for dengue diagnosis

There are several types of tests used for diagnosing dengue infection, starting from traditional tests such as virus isolation in cell culture, serological test, polymerase chain reaction (PCR) to latest advances such as use of biosensors.

Virus isolation in cell culture: Virus isolation in cell culture or live mosquitoes is the conventional test to identify dengue, which was considered as preferred test in last century [13,14]. This test has always been the 'gold standard' for any viral disease. Isolated viruses can be used for virological analysis, which also give molecular epidemiological information. Thus, this test can provide additional data about the patients. Although some published research suggested virus isolation as the best diagnostic test, it is done by using dengue type-specific monoclonal antibodies for immune-fluorescent staining, results show that virus isolation can be integrated as part of the detection process [15]. Cell lines normally used to grow the virus include mosquito cell lines (C6/36 and AP61) or mammalian cell lines (Vero, LLC-MK2 or BHK-21) [16,17]. Virus can also be isolated using intracerebral inoculation of sucking mice. Traditionally, 75 ml flasks were used for confirmation but now 6-well microtitre plates can be used. Virus isolation method requires expensive laboratory equipment and chemicals to preserve the cell line and takes days to weeks to carry out. This method heavily depends on survival of the sample. Hence this directly affects the time frame when the sample can be tested and also timely and proper storage

of the samples are pertinent, as temperature may affect virus viability. Diagnosis by virus isolation is in the range of 20–80%, because only the active virus can reproduce in the cell culture, and greatly depends on specimen collection. However virus isolation remains very useful and relevant as a diagnostic tool, especially for monitoring of dengue epidemiology and evolution as well as determining its antigenic drift. This technique has shown to be more effective than PCR, however, degree of efficiency can be approached only if sera samples are collected before onset of fever [14,18]. Specificity of this technique is only 63% when compared with PCR whose specificity is 100% [19].

Serological testing: Term serology usually refers to the diagnostic identification of antibodies present in the serum and other body fluids. Antibodies such as IgM, IgG and, recently, NS1 are mostly used in serological detection. For primary dengue infection, the acquired immune response starting by 3-5 days is identified by a slow, low-titer increase in the IgM antibody, and the IgG is measured by days 5-7 of the illness. Unfortunately, IgM titers rise much more slowly than IgG titers through a subsequent infection and may result in false-negative results. However, IgG levels rise rapidly in the secondary infection which might be found during the acute stage of the disease. IgG levels can be persevered for years [20]. Conventionally, hemagglutination-inhibition (HAI) assay has been used as a standard to differentiate between primary and secondary dengue infection [21]. However, due to lack of serotyping specificity and unavailability as a commercial test kit, HAI is not used to that extent [13]. IgM antibody capture-Enzyme linked Immunosorbent assay (MAC-ELISA-IgM) is used to detect dengue-specific IgM antibodies present in patient's sera via immobilized IgM by first fixing the anti-human IgM antibody on the ELISA wells [22]. False-positive readings in this test could result due to cross-reactivity of antibody with co-circulating antibodies from other flaviviruses, such as in the serum of malaria or leptospirosis patient and patients with Japanese encephalitis [23].

MAC-ELISA Anti-dengue IgG detection is also carried out by fixing IgG antibody via anti-human IgG, immobilized earlier on the plate wells. However, dengue IgG tests are less specific as compared to IgM because IgG antibody is cross-reactive and has no reserved epitopes, unlike IgM, which interacts only with epitopes of the infecting serotype [24].

The specificity and performance of serological techniques for dengue diagnosis depends on many factors which include:

- Quality of the antigens utilized with ELISA method
- Type of specimen (e.g., whole blood or saliva, serum)
- Dengue serotype and
- IgM and IgG titers

An immuno-chromatography test (e.g., dengue NS1 Ag STRIP Kit) is used for dengue detection. The strip used in this study generally consists of two lines:

- Control line (Biotin-gold colloidal particles coated with streptavidin complex) and
- Test line (Monoclonal anti-NS1 antibodies (mAb)-NS1 Ag-gold colloidal particles coated with anti-NS1 mAb complex).

The appearance of both control and test lines indicates a positive result after incubating in a serum sample for 15 min (assay time). Although this test seems to be simple to perform in any laboratory and offers an excellent specificity, the detection sensitivity of this

test depends solely on the dengue serotype and number of infections [25,26]. Dengue NS1 Ag STRIP Kit is less accurate, sensitive as compared to PCR test [25]. As a result of prior infection or vaccination (e.g., Powassan/Deer tick virus or Japanese encephalitis virus), serological tests with IgM and IgG are performed. These tests require two specimens of sera (taken in the acute and convalescence phases) [27]. Due to these limitations, more than one test (e.g., NS1 antigen ELISA, IgM antibody ELISA and/or rRT-PCR) needs to be performed to ensure the presence of disease in the patient's serum [27,28]. NS1 antigen test should be used as a complementary test since sensitivity and specificity of dengue diagnostics can be enhanced when the NS1 antigen test is performed with the IgM capture ELISA [26,29].

Polymerase Chain Reaction (PCR): Polymerase chain reaction (PCR) is a technique of amplification of DNA from a target RNA to produce cDNA through reverse-transcription reaction and hence also known as reverse transcriptase-polymerase chain reaction (RT-PCR). It has been developed globally for robust, sensitive detection of dengue and other infectious diseases. This method is simple, sensitive, rapid, and if standardized correctly, can be used for detection of genome in human clinical samples, autopsy tissues or mosquitoes, biopsies. These methods differ in terms of the amplified gene regions of the genome, the way they detect RT-PCR products and virus typing methods. Many of the RT-PCR protocols claimed to diagnose and identify dengue serotypes in clinical samples [30-40]. According to the WHO, PCR is a powerful method used for dengue infection diagnosis, but it still needs to be standardized correctly. RT-PCR being more sensitive technique, has advantage of processing a large number of samples at once and can be used both qualitatively and quantitatively.

One of the important factor for the success of the PCR protocol is to utilize the most conserved coding region. But due to instability of the viral genome, it is difficult to identify the true conserved coding region. Regions such as capsid (C), prM, envelope protein (E) and non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) present in the dengue genome have been proposed for the PCR technique [40,41]. The 3'-noncoding region is proposed as the most conserved sequence for serotypes and serotype-specific detections [20]. Other factors that have a strong influence on the PCR sensitivity are PCR parameters, performance of enzymes and the quantity of RNA used for the Reverse transcriptase step [20]. Gamma irradiation also has considerable impact on the sensitivity of the PCR. When 2050 bp RNA sequence were used in a Nested RT-PCR, effect of this radiation was observed while no remarkable damage was observed when specimen of <600 bp was treated [42].

Some of the RT-PCR protocols contain PCR inhibitors (antibiotics and hemoglobin) which might result in loss of sensitivity due to their direct conjugation with DNA or DNA polymerases [43,44]. Another important feature of PCR is its ability to identify the dengue serotype responsible for the ongoing disease. Combination of RT-PCR and digestion of restriction enzyme of amplified DNAs have been used for the development of a fast, simple virus identification method. False-positive results and cross reactivity can be overcome by excluding sequences that are mutual in both the Dengue genome and the DNA or RNA of the human, mosquito [45,46].

Most of the RT-PCR protocols suffer from two problems, absence of a standard protocol and false-negative result due of the variation in the dengue serotypes. In addition, PCR can detect infection only in the early phase and is not efficient after 5-7 days. Use of RT-PCR for dengue detection is not convenient in an endemic region, because it

requires infrastructure laboratories, apparatuses, costly reagents and specific training.

Use of biosensors: Use of biosensors in diagnosis of dengue infection is newer technology which is rapid, sensitive, specific, qualitative and quantitative. This technology is under development and may have desirable traits of being portable, automatic and easily disposable. Their sensitivity and specificity is yet to be fully validated and currently do not fulfill the basic requirement of rapid diagnostic test as gaps exist with regards to their availability, affordability and field applicability as a point of care test. Generally biosensor kits developed have not met the validity and requirements of a rapid test for dengue.

RNA, cDNA, IgM, IgG, Glycoprotein-E, NS1 protein and viral particles are used as different analyte probes. In order to detect these analytes, different types of biosensors are used such as:

Piezoelectric sensors – These sensors work using an oscillating voltage at the resonance frequency of the piezoelectric crystal and then detecting the alterations in frequency according to the required analyte binding with biomolecules on the crystal face. These sensors are generally classified into quartz-crystal microbalance (QCM), surface acoustic wave (SAW) and bulk acoustic wave (BAW).

Advantages: These sensors are sensitive due to the high elastic modulus. As no monoclonal antibodies are used, they are cost effective.

Optical biosensors – These sensors are based on converting a natural response of surface-plasmon resonance using an optical signal such as the absorbance, fluorescence, chemiluminescence to monitor the alteration in reflected light [47].

Advantages: Large number of samples can be screened concurrently.

Disadvantages: Need microscope equipped with fluorescence filters which are costly. Need other electronics and computer to calculate and quantify the fluorescent signal.

Electrochemical biosensors – These sensors are based on stimulating the analyte by tagging or labeling an important element in an electrochemical reaction. Frequently applied labels include enzymes (peroxidase, glucose oxidase, alkaline phosphatase or catalase), ferrocene or In^{2+} salts, and redox mediators (e.g., $\text{K}_3\text{Fe}[(\text{CN})_6]^{3-/4-}$ or methylene blue) [48].

Advantages: Low cost, high sensitivity, simplicity, flexibility, multiplexing capabilities.

Disadvantages: Requires more time to label target analyte.

New techniques and methodologies using biosensors have been developed to detect NS1 antigen with the aim to develop portable, fully automated diagnostic devices. Some of the non-commercial approaches (based on piezoelectric and optical approaches) reported in the literature provides potential for clinical applications [28]. Among these approaches, an electrochemical assay considers to be the most promising. Electrochemical impedance spectroscopy (EIS) is a sensitive method, can monitor the changes in capacitance or charge-transfer resistance which is associated with targets binding to surfaces of specifically prepared receptive electrode and requires no prior labeling [28]. It has been reported that the use of EIS shows good reproducibility and sensitivity in faradaic impedance which involves analysis of classical antigen-antibody binding events.

Challenges in dengue diagnosis

It is very important to have accurate and speedy diagnosis of the

dengue infection so that it can be differentiated from other diseases such as rubella, leptospirosis as well as other flavivirus infections. This is required for the clinical management of the disease as well as surveillance. Currently there are no specific antivirals or vaccines available for treatment of dengue, symptomatic and supportive management of infection is possible which mostly depends on an early and accurate diagnosis. When diagnosis is not performed successfully, problems are expected. As the simple acute febrile illness is a mild form of dengue infection, the patient might not visit a physician, and dengue cases gets neglected [49]. In fact, a patient with mild infection can experience complication free recovery. Low et al. in his article reported the use of both WHO 1997 and 2009 classification of diagnosis of dengue and concluded that older adults with fever and leukopenia should be tested for dengue, even if other symptoms are absent [50]. In addition, dengue infection is sometimes present with atypical clinical appearances such as hepato-gastrointestinal, neurological, and cardiac presentation and this can remain underdiagnosed. Sometimes, physicians in endemic areas do not take into consideration dengue diagnosis based on specific laboratory criteria but instead uses only a positive tourniquet test [51]. This can lead to an increased incidence of epidemiological dengue infection, unnecessary treatment of false-positive cases because of physiological conditions [52,53]. Nevertheless, as already mentioned, the widely used dengue serology test can lead to false positives in conditions such as rheumatoid arthritis, leptospirosis and can result in incorrect diagnosis of dengue infection [54,55].

Though fluid replacement therapy is useful for treatment of dengue, it still poses a risk if not used rationally. The problem of missed dengue diagnosis is often apparent. This problem is usually due to presence of several conditions with a very similar appearance to dengue infection [56]. There is no doubt that there are still cases of dengue that are first diagnosed at autopsy [57,58]. Lahiri et al. reported that 2/3rd of dengue deaths occurred due to missed diagnosis [56]. If missed diagnosis takes place due to the high fatality of dengue infection, fatality can occur. Diagnosis of dengue infection can also be carried out in specimens kept for a very long period of time [57]. If the physician does not consider dengue infection, or if the patient visits the physician too late then delayed diagnosis can be expected. These conditions are likely to happen in non-endemic areas, such as dengue infection cases in travelers [59]. The fact is also considered that delayed diagnosis means more severe, problematic presentation for case management [60]. It should also be noted that there is a possibility of concomitant infection with dengue which can increase the difficulty of diagnosis. One of the good example of hard-to-diagnose situations are concomitant infections with malaria or dengue [61,62]. Hurdle in dengue infection diagnosis might depend on the experience of the practitioner. Even with a high level of experience and expertise, errors in diagnosis can still be expected. This might be due to the fact that many diseases can mimic dengue infection, and these diseases usually share the same geographical pathology pattern as dengue (Table 2).

There are many factors that become hurdle in the diagnosis of dengue infection. These factors are called as epidemiological triad and include host - the patient, pathogen - dengue virus and the environment.

Host – the patient

Host is the person who presents illness to the physician. First clue for the diagnosis of any disorder has to derive from the patient. In order to obtain this clue, it is essential to know the history of patient and disorder [63,64]. History which provides data on mosquito exposure, duration of the current illness becomes necessary for diagnosis of dengue. The patient often has little knowledge to explain his/her illness to physician

Sr. No.	Diseases	Critical differences
1	Chikungunya	Severe joint symptoms, Fewer hemorrhagic episodes
2	Influenza	Prominent respiratory symptoms, No hemorrhagic problems
3	Acute bacterial pharyngitis	Prominent throat and pharynx inflammation, No hemorrhagic problems

Table 2: Diseases with similar signs and symptoms to Dengue.

due to the nature of tropical disease. Sometimes, the patients are children or elderly, and to get history becomes a task [52,65]. Patients are sometimes brought by their family members or colleagues to the physician. In such cases, direct history cannot be obtained from the patients, and the individuals who get these patients to the physician also do not give useful history. Basic physical examination, the tourniquet test, is considered to be a useful tool to diagnose dengue infection but it cannot differentiate dengue infection from other infections such as Chikungunya [52,53]. This causes the requirement of laboratory investigation for confirmation of the diagnosis [51].

Pathogen – the dengue virus

Highly virulent dengue virus has complex structure which can alter the normal function of host cells [50]. Dengue virus is a hard to detect, very small pathogen [55]. Even though some new diagnostic techniques have been launched, limitations of these methods remain apparent [66,67]. Widely used tests such as serological tests, dengue IgM or IgG tests, are not direct diagnosis test to see the presence of virus, but they focus on host response, cross-reactivity to other flaviviruses [68,69]. Virus isolation in cell culture test is very difficult to perform and takes a long time. Although molecular diagnostic test seems to be highly sensitive and specific, is usually expensive, and hence cannot be implemented in dengue-endemic areas which are usually poor, developing countries [70].

Environment

Environmental factors causes difficulty in dengue infection diagnosis. In endemic areas, the main problem is limitation of diagnostic resources. The nature of endemic areas tropical geography leads to difficulty in performing primary diagnosis of dengue infection [71]. As dengue can be misdiagnosed as other tropical diseases, there are chances that other tropical diseases can also be misdiagnosed as dengue infection. Also, concurrent infection with dengue and other infections like malaria and leptospirosis are possible. Travelers returning back to their homes from endemic areas might bring the disease with them [63,64].

Apart from above mentioned factors, there are some laboratory errors which could also make diagnosis of dengue infection difficult. Since, definite dengue diagnosis has to be based on the laboratory investigation, laboratory investigation control is considered to be the main key to generate most useful laboratory results for patient management [72,73]. Error in laboratory analysis is most common [73]. Three kinds of laboratory error in dengue infection diagnosis can occur during pre-analytical, analytical and post-analytical phases of diagnosis (Table 3).

Although, there are several new advanced laboratory investigation tools available for dengue diagnosis, still problems can be observed. Challenges in diagnosis of dengue infection not only occur due to laboratory errors but also due to certain limitations of methods used for dengue diagnosis.

Methods such as virus isolation in mosquito cell lines and live mosquitoes, IgM capture ELISA, dengue-specific monoclonal antibodies, and PCR have major advances in dengue diagnosis, they

have some limitations as well such as Virus isolation technique is a time consuming process, requires expertise and facilities, do not differentiate between primary and secondary infection. Method of detection of IgM antibody requires proper timing. This technique is confounded by false-positive reactions, long persistence of IgM antibodies and requires two or more serum samples. PCR requires specific laboratory equipment and facilities as well as extensive evaluation of the different protocols under field conditions. Commercial kits need to be evaluated critically. Availability of these kits and other reagents, the costs need to be addressed.

Opportunities and future prospects in dengue diagnosis

In coming future, dengue infection will continue to be an important public health problem worldwide. The diagnosis of dengue infection will still remain as the most important issue in clinical management of dengue cases. Researchers all around the world are continuously working towards development of cost effective, portable, easy to use, sensitive, and specific diagnostic tools corresponding with the expanding importance of the disease. Quality management for optimum control of the laboratory investigation process and awareness enhancement of the possible inaccuracy in diagnosis of dengue infection will still remain as the two main focus issues for helping to improve the diagnosis and treatment of dengue infection.

In order to develop an ideal diagnostic technique or tool for early detection of infection, following aspects needs to be given greatest attention.

- (a) Development of tests for early clinical diagnosis of individuals
- (b) Development of serological tests able to differentiate between dengue and other flavivirus infections and even more specifically to determine the infecting dengue serotype
- (c) Development of easy, inexpensive protocols for genomic characterization and viral load
- (d) Need for modifications of existing protocols that simplify specimen handling and transportation
- (e) Development of tools that can suggest a prognosis
- (f) Development of recombinant antigens as a tool for test evaluation

Along with above mentioned aspects, it is also necessary to have greater reagent availability, standard reagents such as antigens, antibodies (monoclonal), cell cultures, positive and negative control sera for protocols standardization in endemic regions and for improving the quality and quantity of the proficiency test.

Researchers working on dengue diagnosis at University of California are planning to develop low-cost viral and antibody assay to detect dengue infection that will not only ensure rapid response and allow timely surveillance but also will promptly diagnose severe cases and will avoid fatal progression of disease. An affordable and accessible tool for identification and surveillance will enhance case triage management, and improve diagnostic capabilities of local laboratories and show significant improvement to accessibility, sustainability and

Pre-analytical error	Analytical error	Post-analytical error
Pre-analytical phase deals with specimen collection and transportation to specific preparation for laboratory investigation procedures.	Analytical phase covers all diagnosis processes using medical laboratory tools.	Post-analytical phase deals with the short period from validation of the analytical result to passing the result to the physicians for further usage in management of the patient.
Most common type of error.	Can result due to human error.	Last phase of the diagnostic cycle, hence can be easily neglected.
Can occur in any clinical setting and become an important factor affecting the quality of diagnosis.	Can occur due to a careless medical scientist. Systemic error can occur due to a problem with the analyzer or systemic error due to the reagent.	Can occur due to a careless medical scientist.
Accounts for 2/4 th of overall error.	Accounts for 1/4 th of overall error.	Accounts for 1/4 th of overall error.
E.g., Misidentification of the patients in specimen collection, inappropriate amount of specimen, mislabeling of the specimen tube, loss of specimen during transportation, improper specimen preparation, and clotting in the blood sample etc.	E.g., Error due to use of automated hematological analyzer in detection of thrombocytopenia, false-positive results of analyzer due to the aggregation of platelets.	E.g., Validation of results is usually problematic for the new diagnostic approaches for dengue infection like molecular-based approaches.
Remedy: Set guidelines and standards, standardization for sample collection using filter paper, use of a double-checking system at all steps of pre-analytical phase.	Remedy: Implementation of quality control and quality assurance in medical laboratories, Regular quality management of the analytical process and regular maintenance of the analytical tool, Continuous monitoring and improvement, rechecking of the blood film.	Remedy: Rechecking of the result before reporting or validation, use of double-checking systems is at all steps of post-analytical phase.

Table 3: Laboratory errors in diagnosis of dengue infection.

cost savings [74]. The Institute of Bioengineering and Nanotechnology (IBN), Singapore of A*STAR has successfully developed a disposable paper based device that will allow detection of dengue-specific antibodies easily from saliva within 20 minutes. The ability of this kit to differentiate between primary and secondary dengue infections makes it a valuable early diagnosis tool for ensuring timely treatment and proper care of patients. They have used an innovative stacking flow design to overcome key hurdles faced by existing lateral flow designs, such as those used in pregnancy test kits. This device is currently under further development process for commercialization and known as Made-in-Singapore rapid test kit to detect dengue antibodies from saliva [75]. There are more than twenty dengue NS1 antigen detection tests, more than thirty IgM commercialized kits, more than twenty IgG and IgA commercialized kits and about seven-eight combination tests (NS1+IgM+IgG) on which research is going on and soon will be available in market.

By taking into consideration the impact of dengue infection in coming future, research on high priority and alert is going on all over the world. Soon we can expect an ideal diagnostic device which can detect dengue at early stages of infection and will ultimately play crucial role in controlling and saving lives of so many population.

Conclusions

Dengue infection is known as one of the world's worst deadly diseases to threaten human life from some decades. Dengue might not show early symptoms, or the symptoms might be similar to other virus diseases. Since, dengue infections may present as a severe disease generally characterized by hemorrhage and shock and because of the absence of an efficient treatment or vaccine for this disease, it is essential to have a consistent, rapid diagnosis and, if possible, to identify the serotype involved in the infections. As there is no marker to predict the progression from classic dengue fever to the severe forms of the disease, correct diagnosis and serotype identification remains of greater importance.

Till date, dengue infection is mainly diagnosed by methods such as isolation of the virus, serology, or molecular diagnostic. These methods are exhausting and require a large amount of time. The Polymerase Chain Reaction method, even though is fast technique for diagnosis in

the early stage, it requires high technical skills and contamination from non-template PCR. Although several commercial kits are available in market for the dengue diagnosis, still a concern with the performance characteristics of these kits exists. When tests require the identification of the virus/viral genome, needs specialized laboratories and become expensive. Affordable commercial kits with adequate sensitivity and specificity to diagnose dengue infection have not been developed yet. Advances in molecular, serological diagnostic techniques have greatly improved the sensitivity and specificity of dengue diagnosis. In coming future, successful application of these techniques are expected to contribute greatly to the etiologic investigation, clinical treatment, and control of dengue virus infections.

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