

EXPERT OPINION

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New development in the diagnosis of dengue infections

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Introduction: Dengue is of major concern around the world. Having no pathognomonic features that reliably distinguish it from other febrile illnesses, laboratory diagnosis is important for confirmation. Ideally, a dengue diagnostic test should be sensitive, specific and applicable from the onset of disease to 10 days post-infection.

Areas covered: In this review, the pro and cons of currently available diagnostic arrays as well as evaluations that have been conducted by numerous groups using both in-house and commercialized kits were assessed and reviewed. We also probed into the challenges and hurdles of applying these assays worldwide. This review also glimpsed at newer technologies that may be invaluable in the future of dengue diagnostics.

Expert opinion: To diagnose dengue, an understanding of the complex immune responses and the clinical features of this disease is essential. The MAC-ELISA currently remains the assay of choice but needs further evaluation and confirmation. Viral RT-PCR and NS1 have gained interest but their inconsistencies and great variability are of concern. Combinations of these tests have improved sensitivity but specificity issues still exist. Consequently, the favorable method of diagnosing dengue currently is to run multiple tests or obtain a paired sample so that more than one parameter is detected or a rising titer is demonstrated.

Keywords: dengue, diagnostics, evaluate, new developments

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1. Introduction

Dengue, an arthropod-borne disease remains a major health concern especially in the tropics and subtropics [1] even though it has been present for centuries. About 3.61 billion people are living in dengue 'at-risk' areas [2]. An estimated 36 million cases of dengue fever (DF) and 2.1 million cases of severe dengue is believed to occur annually [2]. The mosquito species, *Aedes aegypti* and *Aedes albopictus*, are the main vectors in the transmission of dengue viruses. The dengue viruses, of the family *Flaviviridae*, comprise of dengue virus (DENV)-1, DENV2, DENV3 and DENV4. The dengue viruses are positive single-stranded RNA viruses with a 11 kb genome that consist of three structural proteins [capsid (C), membrane (M) and envelope (E)] and seven non-structural (NS) proteins [NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5].

Dengue has traditionally been classified as DF, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). However, recently the WHO has suggested a revised classification that includes dengue with or without warning signs and severe dengue [1]. The symptoms of dengue usually begin with a sudden onset of fever, rashes, myalgia, arthralgia, nausea, vomiting and diarrhea. This phase, the acute or febrile phase, is followed by the critical stage, the defervescence phase. Patients at this phase have normal or subnormal temperatures, rising hematocrits, drop in platelet counts and may suffer from plasma leakage and hemorrhage.

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Article highlights.

- Laboratory confirmation is of great importance to dengue infection mainly due to the absence of pathognomonic clinical features that can distinguish dengue from other febrile illnesses.
- An ideal diagnostic test for dengue: rapid, simple, with high sensitivity and specificity, able to serotype and differentiate primary and secondary infections; unfortunately such an assay does not exist.
- Dengue diagnosis is divided into two main phases: i) Early phase (Virus detection, viral RT-PCRs, antigen detection) and ii) Late phase (Serological testing).
- The main dengue diagnostics currently would be IgM and IgG detection, NS1 capture assays and various real-time RT-PCRs, each with their own pro and cons.
- Newer technologies are being applied to fine-tune available diagnostic arrays and also to design new assays that fit the ideal test concept (with minimal success as of yet), hold promise for the future.
- The key in the development of a useful diagnostic, especially in dengue, is to understand the immune response and complexity of its pathogenesis.

This box summarizes key points contained in the article.

At this time, complications may occur, where patients may have liver failure and hypotension, with a risk of developing shock. If they are not clinically well managed, patients may die; otherwise, patients recover fully at the convalescence stage. However, dengue has no pathognomonic clinical features that reliably distinguish it early from several other febrile illnesses as well as other closely related flaviviruses. Hence, as clinical diagnosis is sometimes not reliable, it is vital a diagnostic test is conducted in order to diagnose the infecting agent and this will then allow proper management and treatment of the disease.

To further complicate dengue diagnosis is that, when one is infected, he/she develops full immunity toward the particular infecting serotype and not toward the other three serotypes. Despite vigorous and intensive research efforts around the world, the understanding of dengue pathogenesis remains obscure and controversial. There are a few postulated hypotheses regarding dengue immunopathogenesis, which include first, the viral factors such as infecting viral serotype and viral loads. Second, the host immune and genetic factors are also believed to be involved in the manifestation of severe dengue. Most of these postulated theories (antibody-dependent enhancement, inappropriate memory cells response and original antigenic sins) revolve around secondary infections with a different serotype than that of the first. However, we will not be discussing these in detail as comprehensive reviews [3,4] on this topic have been done previously. Nonetheless, primary and secondary infection statuses play important roles in dengue diagnosis. Therein, in order for these tests to be effective, some degree of confidence in the test is essential and to do this, dengue diagnostic assays are designed with the intention of improving disease management, which includes early

diagnosis of dengue and for detecting signs of severity. In the absence of a vaccine and antiviral therapies for dengue, early diagnosis is important for timely clinical intervention, etiological investigation and for disease control [5]. With the possible introduction of vaccine in the near future, dengue diagnosis will become even more important, as data from vaccine efficacy trials will determine the usefulness of candidate vaccines.

An ideal dengue diagnostics would be rapid, simple, with high sensitivity and specificity, preferably able to differentiate between primary and secondary infections, as well as to serotype the viruses. Taking into account cultural differences and diverse customs of seeking medical attention when one is sick, the optimal time frame for diagnosis would be from the onset of dengue symptoms to 10 days post-infection [6]. Nevertheless, not all are able to be diagnosed within this time frame, as i) some people consult the physician only when in dire situations, ii) many people in third world countries rely heavily on traditional healing, iii) 2% of world population do not seroconvert and iv) there is a high number of dengue asymptomatic cases [7]. Therefore, an ideal diagnostic tool should be able to detect genuine dengue cases at any stage of illness.

The dengue diagnostic assays that are currently available are mostly serological-based, nucleic acid-based and antigen-based. Despite the many efforts to create a single assay that could confirm dengue, that goal has not been reached. As to why such a diagnostic assay has not been developed, would lie in the complicated pathogenesis of dengue and the fact that multiple sequential infections occurs in dengue endemic areas. Understanding the clinical conditions of dengue patients is essential for appropriate usage of current dengue diagnostics. In fact, there are two different stages in diagnosing dengue (Figure 1) [8], the first being the febrile phase when the patient is viremic, and second, when patients start to form dengue-specific antibodies. As of today, the most widely used methods would be the serological-based ones, especially in dengue endemic countries, as was observed in a survey conducted among primary care physicians in Singapore [9] indicating that about 96.8% of polyclinic doctors and 67% of private clinic physicians still use the dengue serology (Immunoglobulin (Ig)-G/IgM) tests.

A dengue-infected patient produces antibodies against the virus, and in a primary infection, the dengue IgM appears first, and by day 10 of illness, at least 99% of dengue patients have mounted IgM levels. The IgM can then persist in the body up to 2 – 3 months [1]. Conversely, people who have secondary infections, IgM levels are mounted but stay at low levels as the memory dengue-specific IgG peaks. In areas where different serotypes of dengue co-exist and multiple infections occur, lifelong complications arise in the serological diagnosis of this disease, due to pre-existing antibodies, and lower affinity of these antibodies against the second infecting serotype [10]. The lengthy wait for confirmation and complication via serological assays has prompted the development of diagnostics for detection of viral RNA and proteins. With

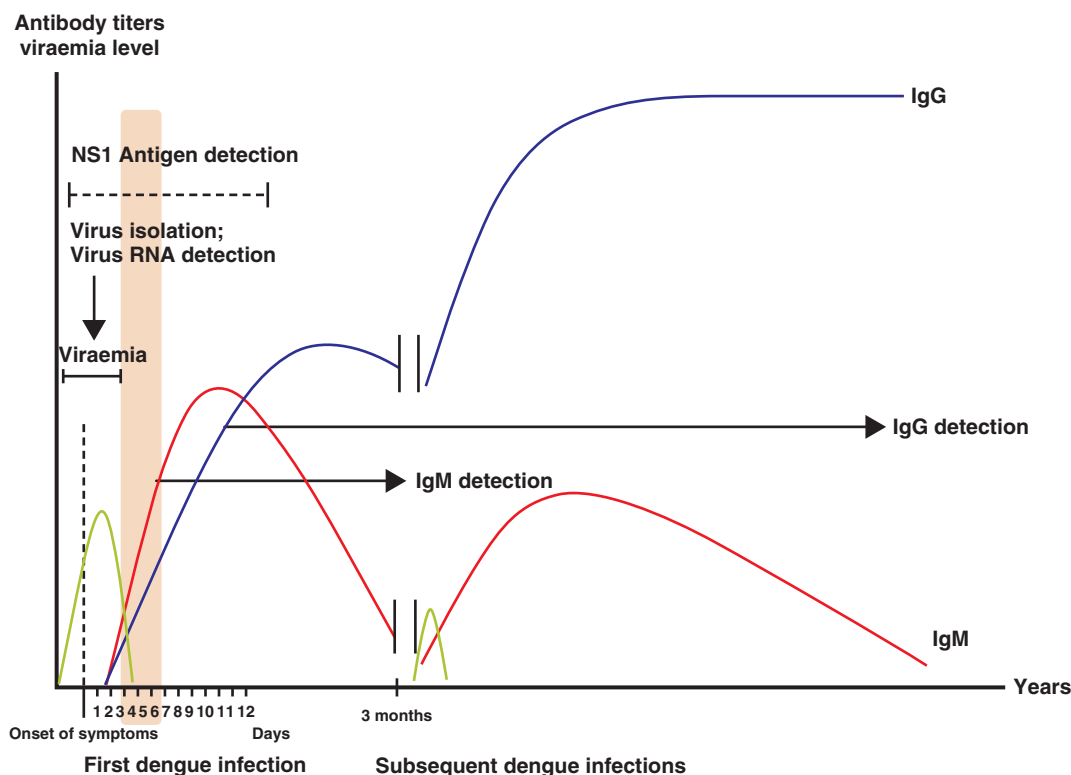


Figure 1. Time course of primary/secondary dengue infections and the suitability of dengue diagnostics at different phases of illness.

the advancement in the polymerase chain reaction (PCR) techniques, many attempts have been made to develop, optimize and simplify new and current reverse-transcriptase-polymerase chain reaction (RT-PCR) diagnostics for dengue. Most RT-PCR developed have target primers that are genus- and serotype-specific [11], and some have been multiplexed for real-time monitoring [12,13] of infecting dengue virus. Another method in early diagnosis of dengue infection would be the determination of dengue NS1 in the blood of patients via enzyme-linked immunosorbent assay (ELISA) method. This is a rapid, sensitive, specific test that is also relatively inexpensive compared to molecular diagnostics assay [8].

2. Viral detection

Virus isolation has always been a gold standard in many febrile illnesses, with highly specific results. In dengue, the most applied cell lines for infection are the C6/36 mosquito cell line, Vero cell line and baby hamster kidney (BHK)-21 cell line. More often than not, sera from dengue patients during the febrile phase are used for virus isolation, nevertheless, the virus has been traditionally isolated from plasma, whole blood and autopsy tissues in dengue cases [14]. A confirmation assay that includes immunofluorescence or RT-PCR is performed once cytopathic effect is noted. Nevertheless, this method is tedious, and takes a long time (7 – 12 days)

before the virus is detected. Furthermore, virus isolation requires cell culture facilities that may not be available in many endemic countries. The method also relies heavily on the virus survival in samples, directly affecting the time frame when the sample can be tested apart from needing very timely and proper storage of testing materials, as temperature may affect virus viability. Despite virus isolation being unsuitable for early diagnosis of dengue, it remains very useful and relevant as a diagnostic tool. It allows monitoring of dengue epidemiology and evolution as well as antigenic drift [15].

3. Serological detection

The serological diagnostics are the most applied methods to diagnose dengue patients in many dengue endemic countries. However, these serological tests are best utilized when dengue virus titers decrease and antibodies start forming. Dengue IgM has been shown to appear from the seventh day of illness onward with 70% positivity and by the tenth day, the patient is expected to achieve 100% positivity of dengue antibodies [16], as the increase in IgM is shown to be directly proportional to the number of days after infection [17]. The dengue-specific IgG would be another serological marker used in dengue diagnosis, along with assays such as haemagglutination inhibition (HI) assays, plaque reduction neutralization test (PRNT), IgM and IgG ELISAs. As of yet, there is no serological

diagnostic assay that can provide definite diagnosis of dengue because they remain presumptive.

One of the earliest diagnostic tools would be the HI assay [18] and it has been the gold standard in dengue diagnosis. This assay uses the ability of dengue antigens to agglutinate red blood cells of geese and anti-dengue antibodies in dengue-infected patients' sera to inhibit such agglutination [1]. The HI assay requires paired sera, making it unfeasible for early diagnosis of dengue. However, it is often used to diagnose a secondary infection from a primary one by the rapidly rising antibodies in secondary infection compared to primary infections. The total antibodies tested in HI does not allow discrimination between infections of other flaviviruses (Japanese encephalitis virus, West Nile virus and Yellow Fever Virus) [1], making it impractical to be used in countries where flavivirus infections are endemic. The HI is also less preferred because it requires serum processing, uses an erythrocyte indicator system, needs to be tested against all four dengue serotype antigens and is extremely tedious and laborious.

One other marker for diagnosis are the neutralizing antibodies formed after a person is infected. These are said to inactivate the infecting virus so that it can no longer infect and replicate in host cells [1]. Neutralizing antibodies are usually specific toward the infecting agent [19], but in dengue, and especially in places where all dengue serotypes are prevalent, sequential infection is known to occur frequently, and the neutralizing antibodies are crossreactive. The PRNT is a manner of measuring these neutralizing antibodies but doing PRNTs require standardization in many aspects including cell lines, virus strains and concentration, incubation temperatures and time, rendering them tedious, labor-intensive, with constant amount of variation by different laboratories, and therefore, a less preferred method of diagnosis.

In place of HI, dengue-specific IgG ELISAs have been developed. The principle behind the IgG ELISA is similar with the IgM antibody capture-ELISA (MAC-ELISA), where instead of using a dengue-specific IgM, a dengue-specific IgG is used in two different formats i) IgG antibody capture-ELISA (GAC-ELISA) and ii) direct measure of IgG antibody bound to fixed antigens [20]. As with the MAC-ELISA, the IgG ELISA also requires paired sera to confirm a dengue infection [10]. The IgG ELISA has a good correlation with HI [21], and is rapid and easy to perform. Using the different avidity of IgG antibodies during primary and secondary infections, the dengue IgG avidity assays were developed, and is useful in determining acute primary and secondary dengue infections [22,23]. Nonetheless, IgG is generally broadly cross-reactive not only between the DENV serotypes, but also within the flaviviruses.

Early diagnosis is important, however, the narrow time frame during which the viremic state of the body declines rapidly as antibodies build up, makes detection of IgM the preferred manner of diagnosing diseases. In dengue, the presence of IgG and low levels of IgM in secondary infections often impede accurate diagnosis using direct IgM ELISA.

Therefore, dengue IgM capture assays (MAC-ELISA) were designed to overcome the antigen-binding competition between IgG and IgM. The MAC-ELISA remains the standard method for diagnosing dengue-infected patients. This assay is simple with the ability to test many samples at one go, and over the years, many laboratories have their own in-house MAC-ELISA [24,25] and commercialized dengue IgM kits have been sprouting. Most of the test kits and in-house IgM capture ELISA have variable sensitivity and specificity (Table 1). The efficacy of IgM kits that comes in multiple formats including microplates, strips as well as cassettes are most of the time not strictly evaluated against well-referenced serum panels [26].

In 2009, a large-scale anti-dengue IgM kit evaluation was conducted by Hunsperger and colleagues (Table 1) [27]. The ELISA kits tested were of IgM capture and indirect IgM detection formats, whereas the rapid diagnostics were of the lateral flow format and particle agglutination format. The evaluation was done using dengue-positive samples and negative control samples of healthy patients and patients with other flavivirus infections, febrile illnesses and systemic conditions. Generally, the commercialized IgM ELISA kit showed sensitivity ranging from 61.5 – 99.0% and specificities of 79.9 – 97.8% at different parts of the world. The rapid diagnostics tests, on the other hand, had lower sensitivities in countries evaluated, ranging from 20.5 – 97.7% and specificities of 76.6 – 90.6%. The vast differences in sensitivity and specificity, among the commercialized kits and also by location, are very disheartening. The major disadvantages of IgM tests are antibody crossreactivity and their inability to identify the infecting serotype. Another limitation to IgM tests would be in the fact that IgM can persist in the body for up to 3 months, and therefore, a detection of IgM only indicates that dengue infection has occurred within this time frame, and remains presumptive [27] unless seroconversion was detected in acute and convalescent paired sera.

For early diagnosis, an accurate, simple and rapid test with good sensitivity and specificity is needed as the normal MAC-ELISA is time-consuming. The Rapid-MAC-ELISA, an innovation to the current IgM test was developed [26] and evaluated. In terms of sensitivity, specificity and reproducibility, the new innovation was highly comparable [26] with the MAC-ELISA. It was also stated to be easy to perform, rapid (within 3 h) and much cheaper than the current available diagnostics assays, making it ideal for usage in resource-limited dengue endemic areas. As for the crossreactivity problem of current MAC-ELISA and diagnostics kits, perhaps with the advancement in molecular technologies such as pyrosequencing and DNA ligase sequencing methods, this may be solved by the development of non-crossreactive DENV antigens and antibodies using recombinant DNA technology [26]. In one such effort, Batra and colleagues have investigated the highly specific envelope protein domain III, to be used as a diagnostics intermediate [28]. A biotinylated recombinant chimeric tetravalent antigen was designed and used in an

Table 1. Sensitivity and specificity of evaluated in-house dengue MAC-ELISA, commercialized dengue IgM ELISA kits and dengue IgM rapid diagnostic kits.

Brand/Test reference	Format	Sen (%)	Spec (%)	Ref.
Kuno <i>et al.</i> , 1987	MAC-ELISA	92.5	97.5	[26]
Nunes <i>et al.</i> , 2011	Rapid-MAC-ELISA	93.0	98.0	[26]
SD Dengue IgM Capture, Standard Diagnostics	MAC-ELISA	75.6	97.1	[99][27]
		97.6	86.6	
Dengue IgM Capture, Panbio	MAC-ELISA	89.5	89.0	[26][27]
		99.0	84.4	
Pathozyme M Dengue Capture, Omega	MAC-ELISA	83.5	86.5	[26][27]
		62.3	97.8	
Pathozyme M Dengue, Omega	Indirect ELISA	61.5	84.6	[27]
Hapalyse dengue-M PA kit, Pentax	Particle agglutination	97.7	76.6	[27]
Dengucheck WB, Zephyr (IgM/IgG)	Lateral flow	20.5	86.7	[27]
Panbio Dengue Duo Cassette (IgM/IgG)	Lateral flow	78.0	81.0	[26][49][27]
		70.7	80.0	
		77.8	90.6	
SD Dengue Duo (IgM/IgG)	Lateral flow	79.2	89.4	[49][48][27]
		53.5	100.0	
		60.9	90.0	

indirect ELISA to detect anti-dengue antibodies in human sera with 100% specificity, but with a need to improve sensitivity [28].

Bearing the need for sensitive, specific, rapid, cost-effective and field-operability in the diagnostics field, especially for low-resource dengue endemic areas, many researchers have delved into developing biosensors for detection of dengue viruses and dengue antibodies [29-31]. Most of the biosensors developed rely on chemiluminescence, which is sensitive, with a low background, a wide dynamic range and needs relatively inexpensive instrumentations [32]. The chemiluminescence-based optical fiber immunosensors (OFIS) are excellent transducers and they have been shown to be more sensitive than using colorimetric and chemiluminescent ELISAs [33]. In one such study, a new diagnostic tool was developed based on chemiluminescent OFIS for the detection of anti-dengue IgM in human serum samples. The assay, which was based on MAC-ELISA, was compared to a colorimetric and chemiluminescent MAC-ELISA. The OFIS was shown to be able to detect low IgM concentration with sensitivity and specificity of 98.1 and 87%, respectively, compared to both other methods, indicating it to be a reliable, simple, fast and cost-effective diagnostic tool [33]. The authors have also suggested that with the possibility of creating recombinant non-crossreactive proteins, improvement could be made to the specificity of OFIS. However, a larger number of samples from different areas of the world will have to be tested using this system to prove that it is indeed as claimed.

Recently, dengue-specific IgA has become a target of interest in dengue diagnosis as the IgA has been shown to appear earlier than IgM and IgG [34]. Besides that, the IgA which rapidly decreases over time makes it a good indicator of a recent dengue infection [35]. Furthermore, the ability to use saliva instead of having to venipuncture patients, for IgA

is secreted in large quantities [35] makes it a promising alternative to other serological diagnosis. An evaluation of IgA ELISA revealed that serum IgA had higher sensitivity and specificity (94.4 and 74.7%, respectively) than the saliva (70.8 and 68%, respectively), however, both remained lower than a conventional MAC-ELISA. A novel immunochromatographic test based on reverse flow technology, MP Diagnostics ASSURE[®] Dengue IgA Rapid Test (Dengue IgA Rapid Test) was evaluated using archived patients' sera [36]. The kit showed 99.4% sensitivity and 99.2% specificity, with comparable detection rates over day of illness with the RT-PCR, in the 179 tested samples [36]. In a recent study, the same kit was evaluated with an overall specificity and sensitivity of 85.1 and 61.0%, respectively, with good repeatability and reproducibility [37] suggesting its use as a simple, useful point of care device where diagnostic facilities are minimal.

4. Antigen detection

Early diagnosis is not only important for better clinical management of dengue patients but also prevents unnecessary usage of antibiotics, allows mitigation of further virus transmission and provides epidemiology data for guidance of health policy decisions once dengue vaccines and antivirals become a reality. The 50 kDa NS1 protein is secreted as a 300 kDa hexamer from dengue virus-infected cells [38] and is said to contribute vastly to different stages of the viral replication [39]. The NS1 antigen has been detected in serum and plasma of dengue-infected patients from the onset of fever up to early convalescence [40] using either NS1 ELISA or rapid diagnostic assay. It seems that in DHF patients the amount of NS1 circulating in serum appears to be significantly higher [41]. Nevertheless, it remains unclear whether this high level of NS1 is a cause of plasma leakage or an effect of

this leakage. Several studies including one from Avirutnan *et al.* (2006), which suggested soluble NS1 could activate complement in the immune system of dengue-infected patients [42] and another from Gutsche *et al.* that suggested that NS1 may actually contribute to endothelium dysfunction by mimicking or hijacking the lipid metabolic pathways [43], therefore indicating that the NS1 may in fact be a cause of plasma leakage in dengue patients.

Being a soluble serum antigen, the NS1 protein has seized an important role in dengue diagnostics [44]. Recently, NS1 ELISAs and rapid diagnostics test kits-based immunochromatography and immunoblotting technologies have been commercialized, not only creating an opportunity for early diagnosis but also becoming useful for low-resource settings as the kits are often lower priced and simple to use [45]. Many of these NS1 kits suggest usage for acute to defervescence stage of dengue (day 1 – 8 of fever onset). The NS1 detection rates were shown to decrease as the IgM levels increase over the days of illness [5,46]. In some studies, the NS1 have been detected in patients' sera up to day 18 of symptoms' onset [47,48]. The commercialized NS1 ELISAs and rapid diagnostics test have been evaluated in many dengue endemic countries (Table 2) [45,46,49,50]. The evaluation of commercial kits is important as the validity and accuracy of these kits need to be assessed, so that candidate kits may be implemented as validated diagnostic method in dengue endemic areas. In 2010, the assessment of two commercialized NS1 assays was conducted in six different countries where dengue is endemic [51] and showed that both kits had poor sensitivity that varied according to the countries tested. In another evaluation study, the NS1 kits tested were less sensitive to DENV-3. Another NS1 kit evaluation by Besoff and colleagues in 2010, on samples from the convalescence phase (day 6 – 36 after the onset of fever) and in PCR-negative IgM-positive patients as well in IgM-negative patients, showed that the NS1 kits had managed to identify 37% of PCR false-negative patients, to resolve secondary convalescence infection as well as late convalescence samples that have declined IgM titers [50]. Then again, the presence of dengue IgG was shown to influence the sensitivity of NS1 detection [45,46].

The evaluations of NS1 kits around the world also showed that the combined use of NS1 antigen and IgM/IgG antibody had improved the overall sensitivity [49,51] of detecting dengue and also the ability to differentiate between primary and secondary infections. This indicates that having an additional dengue NS1 assay along with a MAC-ELISA improves the sensitivity of acute dengue diagnosis and serves as a resolving tool for difficult cases (secondary cases) and for differential diagnosis [50]. Furthermore, in 2011, Chuansumrit and colleagues showed that NS1 protein could also be detected in the urine of dengue-infected patients via NS1 ELISA and NS1 rapid test kits [52]. By classifying patients into DF and DHF, they found that the DHF group had higher levels of NS1 protein in their urine, which could be due to either

plasma leakage or high production of NS1 by infected kidney cells [52]. The ability to detect NS1 in urine is ideal as the method is non-invasive, rapid and does not require specialized equipment. This finding also shows that NS1 has prognostic value, in the sense that it may refer to severity of dengue, and this warrants more study.

With one of the postulated factors in dengue immunopathogenesis being the ability of the infecting serotype to cause a more severe manifestation of the disease [53], identifying the infecting serotype has become an important aspect of dengue diagnostics. Traditionally, identifying the infecting serotype is done by virus isolation and in the last 20 years or so, via molecular approaches. However, virus isolation is time consuming and the newer molecular methods involve costly equipments and experienced technicians. Whereas, using the serological method is challenging as the dengue antibodies produced are crossreactive among each other and also with other flaviviruses. Ding and colleagues had attempted to exploit the NS1 protein for not only dengue infection confirmation but also for serotype identification [54]. The authors developed and evaluated serotype-specific and group-specific NS1 capture ELISAs in their ability to differentiate the four dengue serotype as well as other flaviviruses. This was developed based on the production of epitope-specific monoclonal antibodies. They demonstrated that the four serotype-specific NS1 assays were highly specific in detecting and differentiating the dengue serotypes with no crossreactivity to other related members of the flavivirus. Following this success, the four serotype-specific monoclonal antibodies of NS1 epitopes were combined as a capture complex, to produce the group-specific NS1 ELISA, with identical sensitivity across all four serotypes. This assay was compared with the commercialized NS1 ELISA (PanBio) and was shown to have higher sensitivity [54]. With further evaluation of sensitivity and specificity with dengue patients' sera from around the world, this developed assay may be a valuable asset to further improve diagnostics in dengue.

Although commercial NS1 kits are relatively inexpensive, many resource-limited and third world countries do not have the funding to purchase this valuable acute-stage diagnostic assay. With the aim of further reducing initial costing of producing antigens, Allonso and company developed recombinant polyclonal NS1 antibodies with different refolding protocols that recognized DENV2-infected patient's sera. When the antibody was evaluated using a NS1 capture ELISA, with 43 patients' sera, the antibody detected 100% DENV2 serum and a 60% dengue IgM-positive serum that failed to be detected using commercial kits [44]. With further tweaking of the polyclonal antibodies generated from recombinant NS1, it is possible in the future to develop low cost, sensitive NS1 assay kits, which would be useful in poor dengue endemic areas.

Most dengue diagnostics assays rely heavily on blood and blood products, with a handful that allow other samples such as saliva and urine to be used. This venipuncture method

Table 2. Sensitivity and specificity of evaluated commercialized NS1 diagnostic test kits.

Brand/Test reference	Format	Sen (%)	Spec (%)	Ref.
Pan-E Dengue Early ELISA, Panbio	ELISA	52.0*	90.0	[51]
		72.3	100.0	[46]
Platelia Dengue NS1 Ag Kit, Biorad	EIA	DF: 94.7	100.0	[52]
		DHF: 66.7		
		83.6	98.7	[46]
		66.0*	100.0	[51]
SD Dengue Duo	Lateral Flow	70.6	73.4	[99]
		48.5	99.4	[49]
		62.4	100.0	[45]
		89.9	75.0	[49]
Panbio NS1 Ag Strip Biorad NS1 Ag Strip	Lateral Flow	58.6	98.8	[49]
		DF: 89.5; DHF: 61.1	100.0	[52]
	Wick	89.5	99.1	[46]
		61.6	100.0	[45]
		90.4	99.5	[100]
		72.7	73.8	[49]
Dengue fever IgG/IgM combo device, Merlin Immunoquick dengue fever IgG and IgM assay (Biosynex)	Lateral Flow Wick	79.8	46.3	[49]

is often painful for patients, requires trained personnel for blood drawing as well as other facilities or equipment to further break down blood products. A very recent publication by Muller *et al.* demonstrated a possibility of avoiding the use of blood, but instead to extract circulating biomarkers with minimal invasiveness from the skin [55]. The new method employs microprojection arrays that have been previously used for delivering high molecular weight compounds to the skin. The authors have previously shown the same technology to be able to capture and detect antigen-specific IgG [56]. The NS1 was chosen as it has been shown to be a prognostic marker and it is secreted from infected cells at high amounts. The developed microprojection array-based capture of NS1 and IgG was evaluated and compared to an ELISA using an intravenously NS1-injected mouse model. This is the first report of minimal invasive sampling of NS1 from skin of live mice that does not require sample processing [55]. With further investigation on various aspects such as the effect on mice models that mimics dengue infection, the possibility of multiplexing dengue antigens and antibodies, as well as improving sensitivity and specificity, we can only anticipate the development of such tool into a dengue diagnostics array that may reduce time spent on sample processing as well as to avoid pain in human patients.

5. Nucleic acid detection

Viraemia and antigenemia are observed in patients during the early course of dengue disease [16]. Specimens obtained during first few days of dengue can be confirmed for the infecting agent, not only using the NS1 antigen assays, but also using molecular techniques for viral genome detection. Early detection allows early identification of pathogen, which then enables primary healthcare workers to initiate appropriate

clinical management of patients and also to monitor inpatients for a lapse into more severe manifestation of dengue. In 1983, a new technology, the PCR, was developed, with the ability to amplify a single DNA copy to million copies of the targeted DNA sequence [57]. The method relies on thermal cycling and primer target as template for replication. The PCR has revolutionized the molecular world, and have since the very beginning been used to diagnose diseases such as leukemia, bacterial infections and viral infections. With the incorporation of reverse transcriptase (RT), RNA could be reverse transcribed into complementary DNA (cDNA), and thus the development of RT-PCR, which allows molecular diagnosis of RNA viruses.

The first and foremost step in PCR involves isolating the viral genome from specimens ranging from whole blood to serum, cerebrospinal fluid (CSF), peripheral blood mononuclear cells (PBMC) and autopsy tissues. Many in-house isolation methods and commercial extraction kits exist for the purpose of isolating viral RNA. Traditional methods (TRIzol[®] and Chomczynski-Sacchi technique [58]) are tedious whereas the commercial extraction kits are made to be simple and easy-to-use. However, the efficiency of RNA extraction using various kits have been shown to be different in other viruses [59] and in dengue, de Paula showed the commercial kit to be more efficient in extracting viral RNA [60]. With the emergence of various commercial viral RNA kits, an evaluation on the efficiency in extracting dengue viruses should be performed. This is a crucial step before any PCRs can be done, as the quantity and quality of the extracted RNA reflects what will be amplified using the various PCRs designed for dengue. Without any validation and standardization for first crucial step in molecular diagnostics of dengue, any evaluation study carried out will be inconsequential. Furthermore, current extraction methods require skilled personnel as the likelihood of

cross-contamination is high due to the many pipetting steps involved. In 2011, a self-contained disposable cartridge microsystem was developed to overcome the cross-contamination problem in dengue RNA extraction [61]. This solid-phase extraction-based system was assessed with the conventional extraction method with comparable dengue virus RNA extraction results as low as 1 pfu/ μ l. This system requires further validation especially using human specimens as the initial testing was conducted using cell culture supernatant, which do not reflect the nature of a true human sample of different origins. If the cartridge works as well on human specimens, then an integration of the system with a microchip RT-PCR device [61] would be an excellent tool for point-of-care diagnostics for dengue.

The number of in-house RT-PCRs for detection of dengue viruses has sprouted over the years. The RT-PCR generally has reduced laboratory turnaround time and minimized hazardous contact of laboratory staff with live dengue viruses. Many of the RT-PCRs developed [11-13,62-66] across the world employ target genes of different regions and different amplification techniques. Most molecular diagnostics can be used for serotyping dengue infections, whereas others incorporate quantitative analysis and some have been multiplexed. However, many of the techniques developed have not been commercialized and most have not been under stringent quality assurance [10]. One of the earliest applied techniques in dengue diagnosis was the nested or semi-nested RT-PCR [63,67]. The major concern in this assay would be carry-over contamination. In 1992, Lanciotti used this method to detect and type the four serotypes of DENV [63]. A modification of this protocol into a one-step RT-PCR greatly improved the overall PCR specificity [68]. With the advancement of technology, the ability to perform RT-PCR real time greatly improved the turnover time for dengue diagnostics, and allows for high throughput analyses. The real-time RT-PCR not only enables detection of DENV but also allows quantification where the amplified targets are monitored over time by i) incorporation of non-specific fluorescence dyes (i.e., SYBR green) that binds to any double-stranded (ds) DNA or ii) specific oligonucleotide probes with fluorescence reporter dye (i.e., TaqMan[®] Probes) that only allows detection when hybridized to specific DNA targets. The simplest and least expensive real-time quantitative reverse-transcription-polymerase chain reaction (RT-qPCR) assay would be with the usage of non-specific fluorescence dyes [13,69,70]. The major disadvantage in the SYBR green RT-qPCR would be its ability to bind to any ds-DNA, which includes non-specific PCR products and primer dimers. This could lead to inaccurate quantification of intended target sequence, usually causing an overestimation of DENV RNA concentration. Therefore, when designing primer targets for a SYBR green real-time RT-qPCR, many criteria (e.g., GC content, loop formation) have to be stringently followed, to disallow formation of primer dimers. With the presence of other possible non-target in human clinical samples (e.g., bacterial contamination,

human rRNAs), the primers selected would have to be very specific to the DENV only. The fluorescence reporter probe real-time RT-qPCRs [12,65,66,71-74], on the other hand, are more specific, as they only detect target DNA containing the probe sequence and does not quantify non-specific binding. Nevertheless, primers should still be stringently designed, as dimers have known to compete with amplification of target sequences, and therefore undermining the actual quantity of DENV in tested specimens. In a real-time probe RT-qPCR, multiplexing is possible as long as all the targeted genes are amplified with similar efficiency. However, the use of real-time probe RT-qPCR is still not favored in endemic areas, as it is an expensive method, necessitating specialized equipments and skilled personnel. Often, the quantification method for real-time RT-qPCR, probes or otherwise, uses the absolute quantification by the measurement of cycle threshold (C_t) and compared to standard DENV RNA curve.

The greatest advantage of the real-time assay is the ability to determine viral titer early in dengue illness, enabling physicians to take early course of action in managing a dengue patient. Many of the published protocols for dengue virus detection claim enhanced analytical sensitivity and specificity in detecting viruses. However, an external quality assurance study to monitor the quality and accuracy of dengue molecular diagnostics from laboratories worldwide showed that only 10.9% of the 37 laboratories enrolled had RT-PCR that met all criteria with optimal performance (sensitivity, specificity, serotyping and quantification) [75]. About 80.4% of the labs that applied various RT-PCR protocols and methods need to improve their DENV detection diagnosis procedures. The worrying part is that some laboratories that applied the same protocols had different reproducibility rates [75]. A limitation to the molecular methods would be the presence of false negative (low sensitivity), indicating a need to improve the overall procedure. Another concern would be false-positive results indicating a need to improve specificity and/or to take precaution to avoid cross-contamination. Hence, great precaution is needed when using the semi-nested-PCR/RT-PCR/RT-qPCR to diagnose dengue, where standard operation procedures should be formed and personnel should be trained. Recently, an internally controlled real-time one-step multiplex RT-PCR was developed for dengue diagnosis, in order to provide quality assurance for each experimental step [74]. Despite this, rigorous evaluation of available molecular diagnostics for dengue should be carried out in an organized and systematic manner to improve overall diagnostic performance.

Besides the normal RT-PCR methods, innovations of this revolutionary process has been conducted around the world, for the sole purpose of achieving a diagnostic array that could be useful in the sense of ease-of-use, rapidity, high-throughput, field operability and a fast turnover/turnaround time. The sensitivity of RT-PCR assay can be increased with a combination of RT-PCR and a nucleic acid hybridization assay. The RT-PCR-LH for dengue was developed by

Gunasekara [76], and it showed to improve early detection of dengue. Using the normal RT-PCR [77,78], amplified products were hybridized with a mixture of four DENV serotype-specific DNA probes in liquid phase. Liquid-hybridization is the fastest hybridization format, and the interpretation is done by a size-specific band. Validation of the assay was done using retrospective study samples, showing the RT-PCR-LH to be highly specific with 100% sensitivity and the ability to detect viral genome up to late phase of viraemia [79]. However, using this method requires caution as radioactive materials are used, and proper management will be required. Furthermore, many countries do not have radioactive diagnostics facilities, and many are trying to move away from radioactive substances.

The nucleic acid sequence-based amplification (NASBA) assay is an isothermal RNA-specific amplification assay that has been adapted for dengue virus [80,81]. Developed in 1991 [82], the NASBA uses RT, ribonuclease-H and RNA polymerase as well as two of the target-specific primers. This chemiluminescence technique does not require a thermal cycler, thus eliminating the need for a thermal cycler. Similar to NASBA, the loop-mediated isothermal amplification (LAMP) technology is also an isothermal amplification method that uses strand displacement and amplifying of its stem loop structure in a single temperature, nullifying the need of thermal cyclers. Quantification by this method is done by measuring the turbidity of the sample as magnesium pyrophosphate is produced as a by-product. The reverse-transcription-LAMP was developed for the use of DENV [83]. The technique is rapid, accurate and cost-effective, ideal for use in resource-limited settings [84]. Another molecular diagnostics in dengue would be the transcription-mediated amplification (TMA) where the technology behind TMA, basically involves hybridization of target rRNA by primer containing the promoter sequence for RNA polymerase with a second primer that binds to the DNA generated, and amplification then occurs. The amplicons are then detected using a specific gene probe in a hybridization assay via a chemiluminescence format. The TMA has been used for diagnostics of other infectious diseases and in 2009, Munoz-Jordan has attempted to use this method developed by Linnen [85] based on a similar licensed WNV assay (Procleix, Chiron Corp, Emeryville, CA) in diagnosing dengue. The TMA detected DENV RNA in 80% of acute phase serum specimens that were negative by RT-PCR and a 100% in RT-PCR-positive samples, with an overall performance of about 89% DENV detection rate [86].

6. Biosensors in dengue detection

Biosensors are bio-diagnostics devices that can be quantitative and or qualitative [87]. Biosensors aim to be rapid, sensitive and specific. They are the prototypes of the future rapid diagnostic test kits that will be commercialized if they have desirable traits such as the ability to be portable (for field applicability), automated and easily disposed of.

The development biosensors for dengue is not a new field, as it has been around since early 2000s, and as technology advances, the ability to develop biosensors for dengue has also evolved [30,31,33,88-95]. However, it is important to note that there are details that need to be investigated thoroughly before a biosensor is deemed useful for detection of dengue. One important criterion is purification and chemical modification of complex biological samples, where human genomic DNA may hinder selectivity of viral RNA [87]. The microfluidic (lab-on-a-chip) device and disposable chip-based are some of the available formats for sample preparation [87]. Nevertheless, these devices are not fully compliant with the basic requirements of a rapid diagnostic test as gaps do exist with regard to their in-field applicability, availability and affordability as a point of care test. A survey of the literature shows that most biosensors that are being developed for dengue use one of the three different types of transduction, which are piezoelectric, optical and electrochemical [87]. Among them is an immuno-chip that uses two different monoclonals immobilized on a piezoelectric transducer that was developed to detect glycoprotein-E and NS1 protein [96]. Another used molecularly imprinted polymers to recognize the epitope site of NS1 dengue protein [97]. This circumvents the use of synthesizing monoclonal antibodies and appeared to exhibit good sensitivity and specificity as compared to that using monoclonal antibodies [33]. More recently, chips were developed to perform RT-PCR through specific hybridization with complementary oligonucleotides and these were claimed to be comparable with those of real-time PCR [89,98]. However, this method is prone to multiple interferences (both internal and external) and thus remains to be used in a highly controlled facility. A chemiluminescent optical fiber immunosensor was compared with available MAC-EIA assays for the detection of dengue virus and it exhibited increased sensitivity and good specificity, and would be of interest for diagnosis of asymptomatic [33]. However, it demonstrated poor reproducibility. Other assays utilizing optical means such as magnetic beads, liposomes, reporter probes still require expensive and complex analytical apparatus, complex data processing or other electronic peripherals, thus hindering cost-effectiveness, miniaturizing, mass production and hence commercialization [87]. These techniques also implicate their limited usefulness clinically. The use of electrochemical biosensors is currently time consuming with restricted usage but may be useful where moderate sensitivity is sufficient. Generally, biosensor kits developed have not met the validity/usefulness and requirements of a rapid test for dengue.

7. Conclusion

Serological-based assays are currently still the most popular and despite the many efforts to create a single assay to confirm dengue, that goal has not been reached and this is due to the complicated pathogenesis of dengue and the fact that multiple sequential infections occurs in dengue endemic

areas. Understanding the clinical conditions of dengue patients is essential for appropriate usage of current dengue diagnostics as is understanding the pattern of immune response when developing diagnostics for dengue. Assays that are developed need to be sensitive, detectable as early as possible after onset of fever and have minimal crossreactivity with other circulating flaviviruses. Inter-assay and intra-assay variability needs to be kept at a minimum. In addition, costs, simplicity and rapidity are other factors to be considered during development of a diagnostic test. Future dengue assays are hoped to go beyond confirmation by being serotype-specific, have surrogate markers for disease severity and are capable of showing some indication of immunity/protection that has developed.

8. Expert opinion

Dengue virus is difficult to isolate and propagate, probably as a result of lack of facilities, infrastructure, capacities, skill and also funding. Also the wide geographic serotype and genotype variability needs to be considered when developing an assay. In the development of a diagnostic test(s) for dengue, one has to understand first the complex immune responses to this virus in the context of the host's existing immunity as well as the host's genetic background, the clinical features that may complicate detection, the timing of viraemia, the timing of viral clearance and the advent of antibody responses (both pre-existing and current). Currently IgM remains the assay of choice especially in resource limited and remote areas. This test, however, as a result of the increasing endemicity and its staying power needs to be confirmed as current. Some laboratories carry out virus isolation and here if there is late onset of illness for many reasons may turn out negative as a result of clearance either by pre-existing antibody or a robust innate immune system. Viral PCR is another assay that is performed in more developed laboratories with the necessary skills and facilities and this assay also has its pitfalls in that there is a

narrow window of opportunity here due to its limited appearance in the first few days and its fast clearance by pre-existing immunity. NS1 has gained interest in the last few years but its inconsistency and great variability between laboratories and geographical regions is of concern. Commercial entities have combined some of these tests so as to be able to diagnose dengue irrespective of the day of onset, that is, early in the course of the disease as well as later upon convalescence and great improvements in sensitivity are noted. However, specificity has become of concern as crossreactions are noted with other related febrile illnesses. However, with the advent of globalization, movements between continents have sped up the transmission and spread of dengue throughout the world. Hence in dengue diagnostic evaluations, obtaining a clean negative sample has become a problem, thus complicating specificity. The development of biosensor assays may circumvent this problem; however, these assays have yet to be made easily accessible, convenient, cheap and easily mass produced. It is hoped that newer chromatographic platforms, the use of smaller sample volumes, newer biosensor surfaces for immobilization of markers, nanobiotechnology, mass spectrometry as well as microsequencing will assist toward improving the current dengue diagnostics to an accurate one. With these issues, the way to currently be able to diagnose and confirm the diagnosis is to either run multiple tests or obtain a paired sample so that more than one parameter is detected or a rising titer is demonstrated. The authors feel, however, that there is an urgent need to validate these tests in different regions and assess them to the needs of that region. Assays do not have to perform similarly in all the countries but be tweaked to perform for that region according to their individual needs.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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