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Special report

Issues in contemporary and potential future molecular diagnostics for dengue

Shamala Devi Sekaran; Hui Jen Soe

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, Malaysia.

Corresponding author: Prof. Shamala Devi Sekaran

E-mail: shamala@um.edu.my

Phone: +603-7967 5759

Abstract

Introduction: Dengue has been the most common arbovirus infection worldwide with 2.5 billion people living in over 100 endemic tropical and subtropical regions. Due to the high number of asymptomatic cases and the signs and symptoms being rather unspecific, dengue cases are often under-reported and might influence dengue surveillance programs. Therefore, a rapid, easy to use, inexpensive, and highly sensitive and specific diagnostic tool is essential for early and accurate diagnosis to ease the clinical management of patients as well as for the development of new interventions.

Areas covered: This report discusses the contemporary dengue diagnostic tool, mainly from the aspect of molecular diagnosis where an overview of several nuclei acid amplification tests has been included. Potential molecular diagnostic tools such as biosensor and microarray are also discussed in this report.

Expert commentary: Rapidness and accuracy in terms of sensitivity and specificity is imperative in dengue diagnosis for both clinical management and surveillance of dengue to ensure early treatment and corrective control measures can be carried out. In the next five years it is expected that there will be newer tests developed using not only the lateral flow techniques but more specifically biosensors and nanotechnology. These new technologies will have to be validated with the appropriate number and category of samples and to address the issue of cross-reactivity.

Keywords: Dengue, molecular diagnosis, NAATs, biosensor, microarray

1.0 Introduction

Dengue is an arthropod-borne viral infection caused by dengue virus with 4 distinct but related serotypes (DENV1, DENV2, DENV3 and DENV4) transmitted through the mosquito *Aedes aegypti*. Dengue is characterized by a sudden onset of high fever accompanied by a variety of symptoms such as fatigue, headache, joint and muscle pain, nausea and rash. The clinical manifestations of dengue range from asymptomatic, mild dengue fever to severe dengue, and the complications that arise from the disease include severe plasma leakage, haemorrhage and organ impairment [1]. A second infection by one of the different strains of dengue virus increases the risk of severe complications, however the pathogenesis is still poorly understood and is explained only by postulated theories related to viral virulence factors and host immune responses [2]. It has long been stated that infection with one serotype results in life-long immunity to the same serotype, however, recent cohort studies reported that homotypic reinfection does occur due to an incomplete protection by the pre-existing antibodies [3, 4]. Furthermore, new classes of highly potent neutralizing antibodies against dengue virus infection has also been identified in dengue-specific T cells and B cells [5, 6]. These scientific breakthroughs had rendered some existing knowledge obsolete, thus these issues should be properly addressed before proceeding to newer objectives, especially in drug and vaccine design. However, drug and vaccine development is yet a long and complex process, where current patient management usually involves blood transfusion for significant bleeding or colloids administration for refractory shock,[7] therefore, a suitable but ideal dengue diagnostic test for early

diagnosis is needed at the moment for clinical management, vaccine evaluations and early interventions [8].

2.0 Dengue diagnosis

Most of the commercially available diagnostic tests are tailored to be used in different scenarios depending on the patient conditions and the purpose for which the test is to be used, mainly by the detection of the virus, viral nucleic acid, antigens or antibodies through molecular methods or serological assays. As dengue viruses and antigens are cleared by the immune system after five days of infections, the detection of virus, viral nuclei acid and antigens are recommended for acute samples collected at 1-5 days after fever. On the other hand, samples collected after 5 days of fever should be diagnosed with the detection of anti-dengue IgG and IgM antibodies.[7] Hence, the general approach for dengue diagnosis in current clinical settings is mostly the combination of a molecular method with serological methods, for example PCR with NS1 and IgM detection to cover samples collected at different days of infection. Nevertheless, new techniques and technologies have been developed to fulfil the requirements of an ideal dengue diagnostic test, which is characterized as inexpensive, easy to use, highly sensitive and specific during all stages of infection, providing rapid results, able to determine dengue virus serotype and able to differentiate dengue from other diseases with similar clinical symptoms [9].

2.1 Contemporary dengue diagnosis

Virus isolation using cell cultures or live mosquito larvae has been the gold standard in virological analysis as well as in monitoring dengue epidemiology and the evolution of different serotypes, hence, it is still relevant as a diagnostic tool up to date. Among all the culture methods, mosquito inoculation shown to be the most sensitive method for isolation of dengue viruses, followed by virus isolation through mosquito cell cultures and suckling mice. However, this technique depends largely on the quality of the specimen and it takes more than two weeks for a successful culture, making it impractical to be used as a standard diagnostic technique[10]. Furthermore, its sensitivity and specificity remains not as good as PCR[11] as this depends on the monoclonal antibodies used which may not pick up strains as they evolve. Detection of viral RNA from patient's blood sample using PCR has been used as an early and serotype-specific diagnostic test, however, it is also limited to samples collected during the acute phase of the infection, which is only useful in diagnosing patients from 0 to 5 days after the onset of infection [12]. PCR has been developed as a robust and sensitive tool for many diseases. It is simple, rapid and is able to detect viral genome from different types of samples such as human serum, autopsy tissues and various body fluids [12]. However, the success of PCR depends greatly on the region of viral genome that is being used in the amplification step and hence, the most conserved region for each serotype has always been the region of choice.

Most PCRs developed lack a standard protocol and cannot be adjusted for false negative results due to antigenic variation.[13] It is not efficient for samples collected later than 5-7 days after onset of fever. Furthermore, this assay also requires infrastructure laboratories, apparatus, costly reagents and skilled training which are usually found only in reference laboratories.

Viral NS1 antigen can be detected by enzyme-linked immunosorbent assay (ELISA) or rapid immuno-chromatographic assays up to 9 days following the onset of symptoms, hence, it is more applicable to patients that seek for medical care relatively later in the infection. However, it may be compromised by the pre-existing IgG antibodies found at varying levels in patients with secondary infection especially in hyper-endemic regions [9]. Due to the differential antibody responses triggered based on the immune status of the host, dengue-specific IgG and IgM-based assays can be performed using paired sera collected within a specific time frame to identify seroconversion between acute and convalescent samples [14], as well as to differentiate between primary and secondary infections [15]. IgM detection for single samples, on the other hand, is still useful for dengue surveillance as IgM is expressed earlier than IgG and is detectable after 3 days post onset of fever [12].Anti-dengue IgA antibody has also been recognized as an early high quality serological marker as they are detectable in acute serum that persist for a shorter period of time compared to IgM antibodies. The diagnostic value of IgA antibody detection has been evaluated in different studies, while IgA specific lateral flow test and ELISA have been developed over the decades [1, 2, 3]. The evaluations reported that these tests demonstrated a good overall sensitivity (74-93%) and specificity (73-95%) with a good concordance with IgM ELISA [1, 2]. A meta-analysis also reported that IgA-based diagnostic tests showed a greater accuracy for secondary infections and when the samples were collected 4 days after fever [4]. However, the sensitivity and specificity of serological tests vary greatly between commercially available kits and false-positive results might present in patients with previous infections of dengue or other flaviviruses [9, 16].

2.2 Molecular diagnosis of dengue

Over the years, a large number of molecular dengue virus detection assays have been developed targeting different regions of the viral genome. Nuclei acid amplification tests (NAATs) have been developed for the detection of dengue viruses including reverse transcription polymerase chain reaction (RT-PCR), nucleic acid sequence-based amplification (NASBA), the loop-mediated isothermal amplification (LAMP), transcription-mediated amplification (TMA) and recombinase polymerase amplification (RPA). RT-PCR is amongst the most common method used, where its development has advanced from conventional RT-PCR to real-time RT-PCR based on different dyes and probes including SYBR Green and Taqman probes. Complex multi-step molecular assays have also advanced to multiplex one step real-time RT-PCR, which can greatly improve the timeliness of molecular diagnostic tests, however none of them have been commercialized probably due to

uncertain market potential [9, 17]. The advantages of the real time assay over conventional RT-PCR include rapidity, higher sensitivity and specificity, easy standardization and in addition, able to determine and quantify viral titre early in dengue illness. Although the treatment options for dengue infection are not conditioned by dengue serotype causing the infection or viral titre present in the system, this important information might be useful in the future to allow the physicians to take early course of action in managing dengue patients [18], as high viremia titre, secondary infection and a serotype of DENV-2 has been proposed to correlate with increased disease severity in dengue[19, 20]. The clinical application of these real-time RT-PCR assays greatly depends on the types of samples and for which purpose it is used. The pan-DENV assay using real time PCR or LAMP technique that is able to detect but not distinguish between dengue serotypes was found generally to be more sensitive (93-98%) compared to other assays (72-79%)[21, 22, 23], which is useful as a point-of-need diagnostic method when serotype-specific dengue diagnosis is not required. Multiplex RT-PCR on the other hand, enable serotyping of dengue viruses based on either the distinct melting temperature of PCR amplicons for each serotype in the melting curve analysis during SYBR Green RT-PCR [24, 25] or detected directly using serotype-specific primers and probes in Taqman RT-PCR [26, 27, 28, 29]. Multiplex RT-PCR was found to be as sensitive as hemi-nested RT-PCR, furthermore, Waggoner J, et al (2013) reported that it is more sensitive in detecting samples tested positive only with anti-dengue IgM antibodies (multiplex – 97.2%, hemi-nested – 44.4%) [30]. The development of in-house or commercial real-time Taqman RT-PCR using primers and probes that are specific to different regions of the dengue viruses also allows differentiation of dengue from other flaviviruses. A Taqman-based CDC RT-PCR assay targeting current circulating strains of dengue viruses was the first molecular test approved by the US Food and Drug Administration for the detection of dengue infection among patients with suspected signs and symptoms. It is said to detect clinically relevant contemporary strains of dengue viruses transmitted globally and did not produce false positive results for other flaviviruses [31]. The maximum sensitivity of RT-PCR was observed within three days of fever [32] and was found to be more sensitive than virus isolation and virus titration to detect dengue virus in secondary infection [33]. Paudel D, et al (2011) reported that real-time SYBR Green RT-PCR was equally sensitive in primary and secondary infections while real-time Taqman RT-PCR demonstrated a lower sensitivity in secondary infections [34]. However, the involvement of the thermocycling step in RT-PCR complicates the procedures and hence, it requires personnel with relatively high skill level and the necessary equipment that increases the cost of the assay.

Dengue viral nuclei acid can also be detected by NASBA, LAMP, TMA and RPA, however, these assays are not commonly used as diagnostic tools for dengue as they are less established than RT-PCR. NASBA is an isothermal RNA amplification technique where the assay involves amplification of viral RNA using a set of universal primers at 41°C without the need of thermocycling. The amplified RNA can be detected by agarose gel-electrophoresis [35] or

electrochemiluminescence probe-hybridization [36] while serotyping is possible with the use of serotype-specific capture probes. The development of a real-time NASBA assay using a serotype-specific molecular beacon labelled with fluorophore by Lee, *et al*. (2015) for early dengue diagnosis was shown to have a sensitivity of 88% for samples collected from the first day after the onset of symptoms [37]. It was also proposed to be used for dengue infected Aedes mosquitoes as a virological surveillance tool to predict the outbreaks of dengue [38]. However, the NASBA assay is rather an expensive method and therefore, it is not commonly available in most diagnostic laboratories although it is highly sensitive and specific to dengue viruses [39].

Reverse transcriptase LAMP (RT-LAMP) is described as a rapid, inexpensive, simple and highly efficient isothermal amplification assay that can be used in resource limited settings. It involves a reverse transcriptase step where the RNA samples are converted into DNA, followed by the amplification of DNA in an isothermal condition with universal or serotype-specific primers. The LAMP-created DNA loops can then be detected using a flurophore-labelled probe specific to the loops or DNA dyes that fluorescent under UV light [40]. Studies have been carried out to simplify the procedures by establishing one-step LAMP to include the reverse transcriptase step during the LAMP assay [41] and by developing a single-tube RT-LAMP with a single set or a mixture of primers to detect different dengue serotypes and genotypes, as well as to recognize multiple distinct regions of the viral DNA to increase specificity of the assay [40, 42, 43]. Real-time analysis is also possible for RT-LAMP using spectrophotometry or turbidity meter for colorimetric detection [40]. Similar to other nuclei acid detection method, RT-LAMP is only sensitive during early dengue infection, mainly during 1-3 days after onset of fever [44]. A paper-based diagnostic device utilizing RT-LAMP with fluorescent probes has also been developed by Lo, *et al*. (2013) [45], marking a possibility of developing RT-LAMP as a commercial point-of-care diagnostic tool.

The potential use of TMA as a dengue diagnostic tool has been proposed since 2009 by Muñoz-Jordàn, *et al.* (2009) [46], however, not much studies have been done on the development and evaluation of TMA as a diagnostic tool for dengue. TMA assay is originally employed worldwide for the screening of blood donations for hepatitis B virus, hepatitis C virus, human immunodeficiency virus type 1 (HIV-1) and West-Nile virus [47]. TMA for the detection of dengue viruses has been developed later in the mid-late 2000s and has been used in two studies to screen for possible dengue infection in the blood samples of donors from Puerto Rico [48], Honduras, Brazil and Australia [49]. TMA assay amplifies RNA or DNA samples through RNA transcription and DNA synthesis in an isothermal condition and the RNA amplicons are detected through chemiluminescence-labelled nuclei acid probes [46]. This assay was found to be more sensitive as it detects dengue viruses in most serologically confirmed cases that were tested negative in RT-PCR assay [50].

RPA, an alternative isothermal amplification method has been developed recently as a novel diagnostic tool for the detection of dengue viral nuclei acid [51]. The involvement of reverse transcriptase, recombinase, single-stranded DNA binding protein and strand displacing polymerase in the assay allows rapid detection of both the viral RNA and DNA from clinical samples in a single reaction. The procedure is significantly faster than RT-PCR as it takes only 3-20 minutes to perform the assay [52]. Furthermore, the reaction requires a temperature of 37°C to 42°C which can be achieved by just holding the reaction tubes or auxiliary electricity tapped from a vehicle as demonstrated by Wahed, *et al.* (2014), making it a potential point-of-care diagnostic tool combining RT-RPA with a portable fluorescence-reading device.

2.3 External quality assurance activities

The estimated performance of a molecular test greatly depends on the selection of the reference method in comparing the sensitivity and specificity of the test under evaluation. The performance of serological tests is mostly evaluated by comparing the detection rate of positive dengue infection with a well-established reference method or molecular tests such as RT-PCR. The performance of molecular tests with a similar principle might vary due to the different sequences of primers and probes, as well as the methods and reagents used during amplification [9]. The evaluation of RT-PCR as the most established molecular test involves positive agreement with an existing RT-PCR method or other diagnostic methods such as viral isolation, direct virus titration [53], sequencing of viral genome [31] or NS1 antigen detection [54] using clinical serum or plasma samples obtained from local and global settings [53] or returning travellers [55]. The evaluation of other NAATs methods mainly uses viral isolation or RT-PCR as a reference method to compare their performance and feasibility as a dengue diagnostic test. Most of the evaluations access test performance based on the detection threshold, sensitivity and specificity (Table 1) while some investigate also the positive and negative predictive values of the test [32]. However, the sensitivity and specificity reported in the evaluations also depends on the number of samples evaluated and the strain of viruses used in the development. Not all developed assays evaluated specificity against the related viruses. Thus, false negatives or positives are a concern indicating a need to improve specificity and hence rigorous evaluation should be carried out in an organized and systematic manner to improve overall diagnostic performance.

Table 1: Clinical performance of NAATs methods in term of their detection threshold, sensitivity and specificity.

NAATs method	**Detection threshold**	**Sensitivity**	**Specificity**	**References**
SYBR Green RT-PCR	1 copies/µl	83 – 99%	66 – 100%	[24, 34]
Taqman RT-PCR	10 copies/µl	78 – 98%	74 – 100%	[34, 56]
NASBA	1-10 copies/µl	99 – 100%	96 – 100%	[35, 36]

LAMP	10-100 copies/µl	63 -100%	92 – 100%	[51, 57]
TMA	15 copies/µl	89%	100%	[49, 58]
RPA	10 copies/µl	65 – 96%	94 – 99%	[51]

2.4 Challenges of dengue diagnosis

Flavivirus cross-reactivity highlights an issue in dengue diagnosis as flaviviruses such as West Nile virus, yellow fever virus, Japanese Encephalitis (JE) virus and the recent re-emerging Zika virus share similar size, structural proteins and epitopes in inducing the production of cross-reactive antibodies, therefore causing difficulties in differentiating flavivirus infections serologically [59]. Immunoassays targeting the non-structural protein 5 (NS5) has been studied to improve the specificity of serological diagnosis and showed promising results in differentiating flavivirus as it discriminated between West Nile, yellow fever, JE virus and dengue infections. However, NS5 is present only during viral replication resulting in a shorter detection period [60] and might be masked by other more immunogenic epitopes of the virus. Fortunately, differential diagnosis can rely on molecular detection, as there are remarkable differences noticed between the mapped antigenic relationships and identity of amino acid sequence within flaviviruses [61]. Multiplex real-time RT-PCR and Pan-flavi RT-PCR assays have been developed to detect virus-specific amplicons from flavivirus consensus amplimers located at the RNA polymerase domain of NS5 using newly characterized TaqMan fluorogenic probes and locked-nuclei acids probes[62]. The sequence variability presented in the large amplified region of NS5 gene allows differential diagnosis of flavivirus infections with a specificity of 100% [63, 64], however, the various sensitivities reported were evaluated against a known virus panel and has not been evaluated with clinical samples.

2.5 Potential future dengue diagnosis

Current developments of dengue diagnostic tools mainly focus on 1) the evaluation and improvement of current available diagnostic methods, 2) the development of diagnostic tools based on other clinical markers, 3) the development of new methods for detecting dengue virus and 4) the development of affordable and user friendly point-of-care tests. Studies have been carried out to improve the performance of NAATs, RDTs and ELISAs by either improving the isothermal amplification techniques for NAATs or using the combined antigen and antibody testing approach for serological diagnosis [65]. Many researches have explored into the field of biosensors as alternative technology for the detection of dengue where different transduction mechanisms and electrode materials have been tested in the biosensing platform to improve the capability in detecting either dengue virus [66] or dengue viral nuclei acid [67, 68, 69] as well as to allow the identification of dengue serotypes [70]. Biosensors are said to improve the specificity down to one nucleotide mismatch in the target DNA in detecting multiple dengue infections as they are able to identify

several dengue serotypes on a single sensing platform by selectively differentiating the complementary sequences of each dengue serotype [69, 70, 71]. Furthermore, biosensors are characterized as easy to use, inexpensive, require minimal sample preparation and allow continuous monitoring, making it a promising new diagnostic kit for dengue infection [29]. Such diagnostic tools that are amenable to resource-limited settings would be highly valuable for epidemiologic control and containment during outbreaks.

High throughput multiplex DNA or oligonucleotide microarrays could also be the potential point-of-care diagnostic tool in the future. Thousands of probes for simultaneous detection of several targets can be coated in parallel to allow rapid and selective molecular detection of a panel of clinically relevant microorganisms. Single or multiple dengue virus infections in clinical samples can be detected through specific hybridization of the single-stranded RNA targets with serotype-specific probes coated on the microarray [72]. Probes targeting a panel of arboviruses including Dengue, Chikungunya and West Nile viruses or a panel of microorganisms can also be coated in parallel for the characterization and identification of pathogens in clinical samples with high sensitivity and specificity [73]. Such microarrays target the non-structural proteins, ribosomal RNA and cytochrome genes concurrently to differentiate flaviviruses isolated from different mosquito genera and clinical samples from the infected human [74, 75]. These microarrays can be utilized in clinical diagnosis as a miniaturized portable point-of care tool as well as in arbovirus surveillance programs to screen, analyse and identify arboviruses in infected mosquitoes. However their sensitivity and specificity have yet to be fully validated and currently are not fully compliant with the basic requirements of a rapid diagnostic test as gaps exist with regard to their field applicability, availability and affordability as a point-of-care test. Therefore, future dengue assays are hoped to go beyond and may also include surrogate markers for disease severity.

3.0 Conclusion

An ideal dengue diagnostic test depends on the purpose for which the test will be used. With clinical presentations of acute dengue being rather non-specific, early detection methods are needed for appropriate clinical management and monitoring of patients that lapse into severe dengue. The goal to create a single assay to confirm dengue has not been reached. Newer biosensor techniques, though promising, have also not reached the stage of application. Understanding this disease especially its immune response patterns is essential when developing diagnostics for dengue. Hence assays need to be sensitive, detectable early and have minimal cross reactivity with other flaviviruses. It is hoped that the newer technologies will be able to confirm, be serotype-specific, have biomarkers to predict disease severity and show some indication of immunity that has developed.

4.0 Expert commentary

Rapidness and accuracy in terms of sensitivity and specificity is imperative in dengue diagnosis for both clinical management and surveillance of dengue to ensure early treatment and corrective control measures can be carried out. False positives and false negatives need to be minimized and the underlying disease states need to be understood so that they do not interfere with dengue diagnosis. An important consideration is the expertise of the attending physician, as errors can still be made as there are many diseases that mimic dengue. In hyperendemic areas, dengue can be misdiagnosed as other tropical viral diseases or vice-versa as concurrent infections do occur and dengue might be neglected. We should understand that there are always limitations to any developed assay and for dengue, this would be firstly proper interpretation of the test result, the need for skilled personnel for molecular assays, availability of basic infrastructure for molecular assays, proper critical evaluation of molecular test, unable to differentiate primary and secondary infections, unable to give timely results for clinical management, using the right test depends on when the sample was collected and finally cost.

5.0 Five year view

In the next five years it is expected that there will be newer tests developed using not only the lateral flow techniques but more specifically biosensors and nanotechnology. A number of such tests are currently under validation. Those that have been developed so far have not been validated with the appropriate number and category of samples. More often than not the specificity is usually the one not properly validated as issues with cross reactivity are difficult to validate. Getting samples that do not contain any antibody to flaviviruses is difficult and also the fact that with globalization people exposed to different flaviviruses carry these antibodies and hence may cross react with flaviviruses from other areas when using only serological tests. The new technologies will have to address these issues. One other point to note is what marker(s) to use, i.e. antigen versus antibody or both. Certainly the combo will be best but users will have to note that in some instances they need to repeat the test a few days later if it is negative for the antigen/antibody. With the advent of Zika virus or any other such flavivirus we will envisage problems when using serology and hence for these the best alternative is virus itself or its antigens that are specific to the virus bearing in mind the percent homology between the various antigenic regions of the viruses.

Key issues

- Dengue is a mosquito borne viral infection with clinical manifestations ranging from asymptomatic, mild dengue fever to severe dengue.
- Due to the lack of an established antiviral drug against dengue, an ideal dengue diagnostic test for early diagnosis is needed for clinical management and early interventions.
- Diagnostic tools that are amenable to resource-limited settings would be highly valuable for epidemiologic control and containment during outbreaks.
- The choice of diagnostic test depends on the purpose for which the test to be used, mainly by the detection of the virus, viral nucleic acid, antigens or antibodies.
- RT-PCR is the amongst the most common molecular diagnostic test used in laboratory settings, however, other NAATs such as LAMP and NASBA with proper validation could be potentially the standard molecular dengue diagnostic tool.
- Biosensors and microarrays have been developed as a potential point-of-care diagnostic test with improved sensitivity and portability, however, have not met the validity and requirements of a rapid test for dengue.

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Reference annotations

** Of interest*
*** Of considerable interest*

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