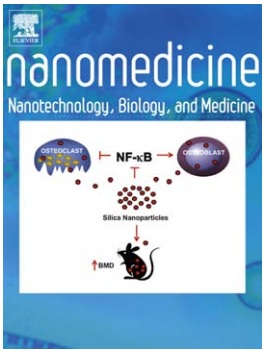

Diagnosing dengue virus infection - rapid tests and the role of micro/nanotechnologies

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Review

Diagnosing dengue virus infection - rapid tests and the role of micro/nanotechnologies

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Diagnosing dengue virus infection - rapid tests and the role of micro/nanotechnologies

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Abstract

Due to the progressive spread of the dengue virus and a rising incidence of dengue disease, its rapid diagnosis is important for developing countries and of increasing relevance for countries in temperate climates. Recent advances in bioelectronics, micro- and nanofabrication technologies have led to new miniaturized point-of-care devices and analytical platforms suited for rapid detection of infections. Starting from the available tests for dengue diagnosis, this review examines emerging rapid, micro/nanotechnologies-based tools, including label-free biosensor methods, microarray and microfluidics platforms, which hold significant potential, but still need further development and evaluation. The epidemiological and clinical setting as key determinants for selecting the best analytical strategy in patients presenting with fever is then discussed. This review is aimed at the clinicians and microbiologists to deepen understanding and enhance application of dengue diagnostics, and also serves as knowledge base for researchers and test developers to overcome the challenges posed by this disease.

Keywords: dengue virus; rapid diagnosis; point-of-care; label-free biosensor; microfluidic platform.

Introduction

Dengue is an endemic viral disease affecting tropical and subtropical areas and is progressively spreading to moderate climates.[1] The global incidence of dengue has grown dramatically in recent decades.[2] More than 100 countries report endemic dengue virus transmission and approximately 2·5 billion people (around 40% of the world 's population) are at risk of infection.[3] It was estimated that more than 50 million infections occur worldwide annually, including 500,000 hospitalizations for dengue hemorrhagic fever (according to the traditional World Health Organization (WHO) classification), mainly among children, with a case fatality rate exceeding 5% in some areas.[4,5,6] However, the true disease burden is poorly known. Recent estimates using cartographic approaches showed 390 million dengue infections per year, of which 96 million are clinically manifest.[7] Today dengue is considered the most important arthropod-borne viral disease causing human morbidity and mortality.[8] Repeated reemergence of dengue in sudden explosive epidemics since the early 20th century has made it a global public health concern.[9]

Dengue virus belongs to the *Flavivirus* genus within the *Flaviviridae* family. It is a positive-sense single-stranded RNA virus, which comprises a spherical particle, 40-50nm in diameter, with a lipopolysaccharide envelope.[1] The genomic RNA is approximately 10·7kb in length and has a single open reading frame (ORF) that encodes three structural proteins, the nucleocapsid or core protein (C), the membrane protein (M) and the envelope protein (E), as well as seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5).[10] There are four distinct but antigenically related serotypes of dengue viruses (dengue virus 1, 2, 3, and 4), each with multiple genotypic variants.[11,12] The geographical and temporal variation in dengue virus genotypes represents a cardinal issue and challenge for the development of dengue diagnostics, and likewise for drug and vaccine development.[13,14] Dengue viruses are transmitted to humans by mosquitoes, principally *Aedes aegypti, Aedes albopictus*, and *Aedes polynesiensis*, some of which are geographically spreading in recent years.[4] *Aedes aegypti* is an important invasive mosquito species widely distributed in tropical and subtropical regions around the world. It is a vector for transmitting several tropical fevers, including dengue fever. *Aedes albopictus* (the “tiger mosquito”) originated in Southeast Asia, but in recent decades has invaded Africa, the Americas, Australia and Europe through the transport of goods and increasing

international travel.[15] In Europe, *Aedes albopictus* has spread, after its introduction into Albania in 1979,[16] along the Mediterranean cost lines and into southern Switzerland,[17,18] southern Germany,[19] and most recently Slovakia.[20] It has also been detected in the Netherlands[21] and Belgium.[22] The presence of tiger mosquitoes in Europe, together with pathogens introduced by international travelers, represents a latent danger for transmission.[23] Vector control would be one of the legitimate options to control dengue. However, present methods and strategies of vector control have achieved only limited success in reducing the transmission of dengue, so some new approaches are now under investigation.[24]

Infection with any of the four dengue serotypes can produce a broad spectrum of symptoms, ranging from inapparent infection, mild dengue fever (DF) to severe and fatal forms – dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[11,25,26,27] In 2009, the WHO classified severe plasma leakage, severe haemorrhage, and severe organ impairment as severe dengue.[4] Typical DF is characterized by high fever, severe headache, arthralgia, myalgia, retro-orbital pain, and maculo-papular rash. The clinical feature of acute dengue infection is non-specific but 5-10% patients progress to the life-threatening forms, resulting in bleeding, low blood platelets counts, and blood plasma leakage, or to DSS, where dangerously low blood pressure occurs.[28,29] Infection with one dengue serotype leads to a life-long protective immunity against homotypic reinfection, but only partial and temporary cross-protection against heterotypic infection.[30] It has been shown that secondary infection with different dengue virus serotypes is a major risk factor for DHF/DSS,[31] probably due to either antibody-dependent enhancement,[32] proliferation of cross-reacting and low-affinity T cell clones termed ‘‘*original antigenic sin*’’,[33,34,35] cytokine storm,[34,36] autoimmune responses,[37,38] or their combination.

Currently no approved dengue vaccines or specific antiviral treatments are available. New vaccines or therapies should be effective against all the four dengue serotypes.[39] A chimeric vaccine based on the yellow fever vaccine strain backbone has been reported to induce antibodies against all four dengue types and has been tested in clinical trial III efficacy studies.[40] Multiple clinical trials showed this tetravalent dengue vaccine was efficacious against severe dengue and reduced hospitalization due to dengue. However, there

was discordance between the neutralizing antibodies in trial participants and the tetravalent protective efficacy of the vaccine.[41]

As a febrile disease, the differential diagnosis between dengue infection and other febrile diseases is essential wherever dengue is prevalent, but is of particular importance in the tropics, where the fever may be mistakenly attributed to malaria or pneumonia and thus lead to ineffective and costly overtreatment and potentially contribute to pathogen resistance to antibiotics.[42,43,44,45] Misdiagnosis is also of risk to the patient, especially if he/she is predisposed to progress to severe dengue. Due to the lack of pathognomonic clinical features that reliably distinguish dengue infection from other febrile illnesses, definite virological and serological laboratory confirmation is required[46] and is critical to limit pathogen spread and alleviate morbidity and mortality.[11,47] In recent years, the progressive spread of dengue has led to a growing demand for rapid and miniaturized point-of-care (POC) tests for this disease, especially in remote or resource-poor settings where the current relatively expensive tests may not be readily available. With evolution of technologies in bioelectronics and micro- and nanofabrication, new methods and tools for dengue diagnosis are being developed and in the process of evaluation. This review presents an overview about established and emerging diagnostic tools for dengue infection, including novel rapid and micro/nanotechnologies-based methods and platforms (Table 1). A historical perspective on diagnostic technologies for dengue is given in Figure 1. With a view on clinical application, the value of diagnostic tests for dengue in patients presenting with fever in different epidemiological scenarios is also discussed.

Overview on currently available assays in research and clinical contexts

Conventional laboratory diagnostic methods for dengue infection

Laboratory tests for diagnosis of dengue infections encompass virus isolation, virus RNA or antigen detection, and serological tests of specific anti-dengue antibodies. Detailed information can be found in

specific reviews.[1,11,48] As a necessary background for future test evolution, existing methods are summarized briefly below.

Isolation of dengue virus by cell culture or from mosquitoes is considered the "gold standard" and provides the most direct, specific, and conclusive approach to diagnosis. However, high-level equipment, technical skills, manpower, and significant time are required to isolate and serotype the virus.[49] Molecular techniques based on reverse transcription-polymerase chain reaction (RT-PCR), such as one-step or nested RT-PCR,[50] real-time quantitative RT-PCR,[51,52,53,54,55] nucleic acid sequence-based amplification (NASBA),[56] or RT-loop-mediated isothermal amplification (RT-LAMP),[57,58,59,60] are gradually being accepted as new standards over virus isolation for the detection of dengue virus in acute-phase serum samples. They provide a fast, serotype discriminating, more sensitive, simple, and reproducible diagnosis for dengue, but are impeded by easy sample contamination and high technological requirements. Antigen-based assays, in particular detection of the dengue NS1 protein, have proven to be a useful means for the diagnosis of acute dengue infections.[10] Current commercial NS1 antigen detection kits do not differentiate between dengue virus serotypes and results can also be compromised by pre-existing virus-IgG immunocomplexes.[61] Further studies are needed to evaluate and validate the performance and clinical significance of NS1-based tests in specific clinical settings. Serological assays are most commonly used to infer dengue virus infection as they are relatively inexpensive, simple, and easy to perform. The IgM antibody-capture enzyme-linked immunosorbent assay (ELISA) format is most widely employed to detect dengue-specific IgM in diagnostic laboratories and commercial kits.[1,62] In addition to serum samples, specific IgM can be detected in plasma, saliva, and whole blood on filter paper, but not in urine. Positive results of dengue IgM-based assays only suggest recent infection with dengue and do not necessarily imply that dengue infection is the cause of the current febrile illness, and negative results do not rule out dengue infection.[11] Dengue-specific IgG detection by ELISA can be used to confirm a dengue infection based on a significant rise in the titer of specific antibodies between acute- and convalescent-phase serum samples. Serology is also widely used to classify dengue infections as primary or secondary, using IgG titer thresholds or the IgM to IgG ratio.[1] Identification of dengue serotypes using IgM- and IgG based assays is relatively difficult due to broad cross-reactivity within the dengue group or/and among flaviviruses.[11] Some reports showed that the cross-reactivity issue among dengue viruses and

the other flaviviruses could be addressed by using designer antigens (so called multiepitope proteins) based on epitopes unique to dengue viruses alone.[63,64] An immunofluorescence assay using infected cells is the only safe method to distinguish dengue serotypes. However, this technique is mastered only by specialized virological laboratories. In addition to the mentioned methods covering the virological, molecular, and serological diagnosis of dengue, plaque reduction and neutralization test and microneutralization assays are used to determine efficacy and to measure protection in epidemiological and vaccine development research, but reproducibility between laboratories is low due to the lack of standardized laboratory materials and methods.[4,65]

The interpretation of dengue diagnostic tests is largely influenced by the dynamics of dengue virus infection (Figure 2).[66,67] Following the initial infection, the dengue virus shows cell specific tropism with rapid replication in immune cells, the liver, and endothelial cells,[68] exhibiting peak viraemia at the time of, or shortly after the onset of symptoms. Intact dengue virus and virus components such as RNA or NS1 antigen can be found in serum, plasma, circulating blood cells, and infected tissues from 0-7 days following the onset of symptoms, which roughly corresponds to the period of fever (Figure 2).[11] During the viraemic phase of the disease, direct virus detection could potentially be used for early, definitive, and serotype-specific identification of dengue infections. The host immune response to dengue infection is the basis for the serological diagnosis. The patterns of serological responses are different when patients experience a primary infection (with dengue or another flavivirus) or a secondary infection (Figure 2).[11,67,69] In primary infection, virus-specific IgM develops following the decline of viraemia and is detected 5 or more days after the onset of illness in most infected individuals, peaking at 2 weeks post infection with a titer decline to undetectable levels within 2 to 3 months. Dengue-specific IgG typically appears within 10-15 days post infection. In secondary infection, IgM appears at the same time as in primary infection or even earlier, but the levels are significantly lower. IgG presents from the previous infection is detectable early in the acute phase followed by a further titer increase. After dengue infection, IgG can be detected over decades, which renders the serodiagnosis of current, recent, and past infections more complex. Based on the kinetics of antibody responses in acute- and convalescent-phase sera, differentiation between primary and secondary dengue virus infections can be attempted, but few tests have proven capable to do so.[1] Dengue-specific IgM alone may be

acceptable for surveillance reports, but is insufficient for patient management and to monitor the efficacy of control measures in a clinical setting.[3]

Collectively, diagnosis of dengue is dependent on the phase of the infection. A suited approach to diagnose current dengue infection is to combine several parameters from virological, molecular, and serological assays or to demonstrate a rising titer in a paired sample. Overall, an understanding of the complex immune responses and the clinical features is important for dengue diagnosis and for a rational therapeutic management.[70]

Current point-of-care diagnosis of dengue by rapid diagnostic tests

Laboratory tests are essential for accurate diagnosis of acute dengue virus infection and subsequent adequate treatment, as well as for patient management. Due to lack of laboratory diagnostic resources in many dengue endemic regions, simple rapid diagnostic tests (RDTs) are urgently needed for POC diagnosis. An ideal diagnostic test for POC applications should comply with WHO's ASSURED (affordable, sensitive, specific, user-friendly, rapid, equipment free, and delivered to those in need) criteria that summarize specification goals for POC testing.[71] Multiplexed tests covering multiple infectious agents are especially needed in the case of diseases with indistinct clinical symptoms or where co-infections are possible, such as in fever with acute onset.[72]

In the past decade, lateral flow (LF) or immunochromatographic strip (ICS) tests have been one of the very few diagnostic technologies that were successfully deployed in the developing world because they require little or no sample preparation, provide rapid and reliable results with no electronic components and thus can be manufactured at low costs and operated by unskilled personnel. A typical ICS includes a sample application pad, a conjugate pad, a nitrocellulose membrane, and an adsorbent pad (Figure 3).[73] The conjugate pad contains gold nanoparticles (AuNPs) conjugated antibodies. Once AuNPs conjugated antibodies encounter the target antigen from the sample, an Ag-Ab-AuNPs complex is formed. This complex then moves to test zone and is captured by second antibodies to the same antigen, resulting in visually

detectable accumulation of red color at the test line. A control line consists of anti-IgG antibody to capture all IgGs, including AuNPs conjugated antibodies, and appears red in both positive and negative test cases. Excess fluid is collected by the absorbent pad. Lateral flow immunoassays (LFIA) have been developed for the detection of dengue virus NS1 antigen, IgM, IgG, and IgA antibodies by several commercial companies and have been widely used for POC diagnosis. In order to prevent substandard performance of commercial dengue RDTs for acute dengue diagnosis and regulate RDT market, large-scale-evaluations funded by independent international organizations such as WHO were performed during recent years.[62,74,75] Guidelines for the evaluation of dengue diagnostic assays have been published and provide a standardized approach to diagnostic assessment.[11,76] A key conclusion from independent assessments published in peer-reviewed journals is that IgM-based RDTs for dengue are not sensitive enough to diagnose acute dengue infection as a standalone test.[77] IgG-based RDTs for acute dengue diagnosis are not recommended. NS1-antigen-based diagnostics are a very valuable POC component for detecting dengue infection at the early phase. Combining dengue antigen- and antibody-based tests has the potential to significantly enhance the sensitivity of acute dengue diagnosis. Despite recent improvements in the RDTs, further investigations are required to explore important practical aspects of dengue RDT performance, e.g. differentiation of primary and secondary dengue infection, prediction of disease severity, the impact of RDT storage conditions, and the optimal sample type. For an in depth review of the current diagnostic evaluations for commercial dengue RDTs, we refer to the review of Blacksell.[67]

Emerging technologies and diagnostic tools for rapid diagnosis of dengue infection

Given the demand for rapid and miniaturized POC tests for dengue diagnosis in the tropical environment, new easy-to-use methods and analytical platforms are being explored as alternatives to classical, "heavy" lab instrumentation for diagnosis. This section summarizes recent advances of these diagnostic tools, including three label-free biosensor methods and two miniaturized platforms based on microarrays and on microfluidic technologies for dengue virus detection.

Biosensors

Biosensors are compact and portable analytical devices that convert a biochemical reaction or interaction into an analytical signal that can be further amplified, processed, and recorded.[78] Recent advances in bioelectronics have generated new techniques for detection of dengue antibodies and RNA, based on biosensor technology. Three recently reported methodologies and devices (quartz crystal microbalance, surface plasmon resonance, and electrochemical impedance spectroscopy) for detection and identification of specific biomolecules related to dengue virus are described here. These label-free methods represent three main types of physical transduction mechanisms (piezoelectric, optical, and electrochemical) for biosensor design, which have attracted considerable attention.[79]

Quartz crystal microbalance (QCM)

The quartz crystal microbalance (QCM) is a physical technique that detects changes in the resonance frequency of an electrically driven quartz crystal resulting from changes in mass.[80] As a label-free and extremely mass-sensitive device based upon the piezoelectric effect, QCM allows the detection of binding events between trace medical analytes and receptors on its surface. Claimed benefits of QCM over other transducer types include better sensitivity, ease-of-use, integration with compact analytical devices, and economy since device production is relatively simple.[81] This renders QCM interesting for POC technology for early detection of diseases. In one study, a QCM-based immunochip for detection of dengue viral antigens was fabricated.[82] Two specific monoclonal antibodies against the glycoprotein-E and NS1 protein, respectively, were immobilized on a piezoelectric transducer and used to detect dengue antigens in buffer. Multi-antibody coating allowed the capture of several different antigens, thus improving sensitivity. The addition of a protein A layer to orientate immobilized antibodies further enhanced sensitivity.[83] Sample pretreatment was optimized, and spiked samples and clinical specimens including five dengue virus serotype 2 (DENV2)-positive and ten dengue-negative sera were analyzed. The resulting immunochips could

distinguish DENV2-positive from dengue-negative samples. Sensitivity was similar to the NS1 antigen ELISA. The immunosensors described in these two papers are characterized by low technical requirements for the test site, easy result interpretation, and rapid availability of results.

In another work, the capture antibodies on the QCM were replaced by a thin film of molecularly imprinted polymers (MIPs) which had specific binding sites for the dengue viral NS1 protein.[84] MIPs are polymerized in the presence of antigen peptide sequences. The peptides are then removed by acidic and alkaline washing steps, leaving molecular imprints that accurately conform to the antigen. Such imprints can work as binding sites and replace capture antibodies. In this study, synthetic NS1 dengue protein specific epitopes were used as templates for oriented MIP imprinting. The imprinted surfaces exhibited highly selective recognition for NS1 dengue protein in clinical samples, documenting the value of MIP-QCM for diagnosing early dengue in clinical specimens.[84] Assay time was less than 1 hour, and the results correlated with the ELISA test and were in concordance with the clinical diagnosis. Samples often do not require dilution or other pretreatments for effective analysis. Replacing monoclonal antibodies by such artificial binding sites may be beneficial for shelf life of the assay and might give better control over specificity.

In another recently published QCM approach, real-time circulating-flow QCM biosensing combining oligonucleotide-functionalized gold nanoparticles (i.e. AuNP probes) was used to specifically detect the DNA fragment, which is amplified from the dengue virus genome, by layer-by-layer hybridization of AuNP probes.[85] Bifunctional AuNP probes act as amplifiers and verifiers to increase assay sensitivity and specificity. As few as 2 plaque forming units (PFU)/ml of DENV2 could be detected in clinical blood samples. Subtype-specific probes tested against all four serotypes responded specifically to the homologous genomic region of the correct subtype. The sensitivity and specificity of this DNA-QCM method are comparable to fluorescent real-time PCR, but this new method is label-free and does not require expensive equipment.

Although QCM chips have been successfully used in protein assays, their application to clinical samples may be limited by serum proteins interference, complex interactions of interfacial parameters, and environmental

interferences.[83,86] Therefore, further improvements that deal with the issue of robustness are goals for future work in this technology.

Surface plasmon resonance (SPR)

Surface plasmon resonance (SPR) is an optical technique that is sensitive to refractive index changes occurring in the immediate vicinity of a sensor surface upon analyte binding. It is an optoelectronic phenomenon where light incident on a metal surface at a given angle can excite surface-bound electromagnetic waves, surface plasmons, which propagate along the interface between the metal and the ambient medium. Unlike QCM, SPR is not affected by water associated with the adsorbed layer nor by conformational changes in the adsorbed species.[87] Associated with the surface plasmon is an evanescent field that probes local changes induced in the refractive index of the ambient medium upon analyte binding, resulting in a measurable shift in the angle of incidence at which SPR excitation occurs. Monitoring this shift as a function of time yields a sensogram containing binding event information.[88] SPR biosensors have become a mainstream method for biomolecular interaction analysis.[89] Advantages include the fact that they are label-free, allow real-time monitoring, require only minute samples, have a high throughput, and exhibit remarkable sensitivity. A SPR based immunosensor for serological diagnosis of dengue was developed, which combined the selectivity of molecular recognition and the high sensitivity of SPR signal transduction, resulting in a single step, label free, and real time assay.[88] The dengue virus antigen was attached as sensing element in a self-assembled monolayer on a gold chip surface. Presence of dengue virus specific IgM antibodies in patient serum was monitored by increase in resonance angle within direct immunoassay, whereas this biosensor could also be used for detection of dengue virus antigen using indirect competitive inhibition immunoassay.[88] In a further study, a rapid immunoglobulin M (IgM)-based dengue diagnostic test using a SPR biosensor was proposed for rapid, 10-minute POC detection of dengue virus antibody in human serum samples (Figure 4).[90] Four dengue virus antigen serotypes were used as ligands. Validation tests in 30 dengue IgM positive patient sera including high, mid, and low antibody titer samples and 22 dengue-negative sera showed that the sensitivity and specificity of this proposed SPR biosensor was 83-93% and 100%,

respectively. With only 1 μl of patient serum an SPR angle deflection was detected to determine the ratio of each dengue serotype. Another recent paper reported a technology variant, Localized Surface Plasmon Resonance (LSPR) to diagnose dengue using an all-optical fiber sensor exploiting specular reflection from gold nanoparticles.[91] Dengue anti-NS1 antibody was immobilized on AuNPs deposited on the end face of a standard multimode fiber. This sensor detected NS1 antigen down to a limit of quantification of 0·074 μg/ml, which was in the range of serum NS1 antigen concentrations (0.04 to 2 μg/ml) detected by a sensitive capture ELISA in patients during the acute phase of the dengue infection, and presented its potential for early diagnosis of dengue.

SPR technology has many characteristics that render it well suited for POC testing. It enables quantitative, label-free, and real-time sensing of a wide range of clinically relevant targets within seconds and with high sensitivity and specificity. However, in the context of a POC testing configuration, field applications of SPR have been hindered by the fact that detection devices are still bulky.[92] Further miniaturization of optical and electronic components is therefore a development goal for SPR-based devices.

Electrochemical impedance spectroscopy (EIS)

A growing number of recent commercial and experimental biosensors rely on electrochemical transduction, i.e. the direct measurement of currents and/or voltages to detect analyte binding. The absence of optical components and the continuous progress in electronic components miniaturization is an advantage over other transduction schemes, and miniaturized, high sensitivity, and low power devices can be achieved, which may result in low cost devices if large numbers of sensors are produced. These evident advantages for POC diagnosis call for more specific efforts toward early detection of infection and deployment of such tests in the field.[93]

Electrochemical impedance spectroscopy (EIS) probes the interfacial properties of a modified electrode by monitoring electron transfer resistance at the electrode surface caused by the adsorption and desorption of analytes. EIS allows detection of biomolecular binding signals using molecular labels, but can also be used

for analysis of DNA, proteins, and bacteria in a label-free manner. Sensitivity of EIS benefits from optimized immobilization of capture biomolecules on sensor surfaces.[94] Employing antibody-conjugated-nanoparticles[95] or aptamers[96,97] on the sensor surface can significantly enhance performance. As EIS is non-destructive, it can monitor ongoing time-varying processes.

Recent advances in EIS applications result from advances in instrumentation and the rapid progress in microfluidics technology. EIS as a label-free, simple, sensitive, and cost effective electroanalytical tool has been used for the detection of bacteria and viruses, including dengue virus.[98] An EIS biosensor was fabricated using gold electrode immobilized concanavalin-A (ConA) by means of gold nanoparticles and polyvinyl butyral for probing glycoprotein patterns in blood during dengue.[99] The interaction between ConA lectin and patient sera in DF and DHF showed a clear increase of the electron-transfer resistance with DF or DHF sera compared to dengue negative serum. The patterns of impedimetric responses may allow the distinction among sub-classes of an immunological response in dengue infection and could be used to predict and monitor the course of the disease once diagnosed. In a more specific approach, binding of DENV2 viral particles to its serotype-specific IgG antibody were measured by EIS on a nanoporous alumina membrane.[100] The detected linear relationship between pore resistance and DENV2 concentration allowed quantitative detection of DENV2, and nanochannel capacitance could quantitatively indicate specific virus-antibody binding within nanochannels. Such a sensor with EIS readout was tested for detection of dengue.[98] The change in pore resistance exhibited a good correlation with virus concentration from 1 to 900 PFU/mL with a detection limit of 0·230 PFU/mL for DENV2 and 0·710 PFU/mL for DENV3. This sensitive and label-free sensor yields a result within 40 min. The small piece of alumina membrane allows it to be easily disposed or autoclaved. These characteristics are well suited for POC use in the field.

An EIS DNA biosensor based on a nanoporous alumina membrane was developed for detection of dengue virus oligonucleotide sequences.[101] Probe DNA sequences were covalently attached inside the membrane pores and binding of synthesized dengue target sequence to the probe caused measurable impedance changes due to pore blocking, exhibiting a linear response to the concentration of target sequence in the range of 10^{-12} to 10^{-6} M, and a very low detection limit ($2{\cdot}7 \times 10^{-12}$ M) for the 31-mer complementary analyte. Single base

mismatched (MM-1) strands and non-complementary strands could be differentiated. The development of inexpensive, selective, and sensitive DNA biosensors for dengue virus detection in the field is therefore at the horizon.

It has been repeatedly demonstrated that protein and DNA binding to immobilized probes is detected by measuring impedance changes at electrode-solution interfaces, but most published reports use analytes which are in principle of real-world interest but are tested in highly purified conditions. Much effort is still required to bring about robust analysis of clinical samples using impedance biosensors that will enable point-of-care applications.[78,93]

Micro- and nanofabrication

Advances in micro-and nanofabrication technologies have greatly contributed to improving medical diagnosis in the lab and at the POC in the past decades.[46] Benefits of miniaturization include low consumption of costly reagents and power, minimized handling of hazardous materials, short reaction times, portability and versatility in design, and capability for parallel operation, all of which are particularly important for POC diagnosis in the tropical and developing countries where dengue is endemic.[102] Microarray and microfluidic technologies are therefore discussed in the following paragraphs.

Microarray technology

Microarrays are sensor arrays coated with different probes for simultaneous detection of multiple targets. Thousands of biomolecular interactions can be probed in parallel.[78] Miniaturized sensor arrays can offer multi-component information, dynamic compensation for sample matrix effects, detection of malfunction/deterioration, and improved selectivity through signal pattern analysis. Recent progress has brought microarrays to the forefront of clinical diagnostics and medical research. DNA microarrays appear suited for highly parallel detection and identification of microorganisms from clinical samples[103] and

therefore promise more rapid, accurate, and cost-effective detection of pathogens compared to culture techniques or conventional immunoassays.

A low density microarray for the detection and identification of seven pathogenic flaviviruses, including yellow fever (YF), West Nile (WN), Japanese encephalitis (JE), and the dengue 1-4 viruses, has been reported.[104] Amplification targeting five different regions of the seven viruses in parallel was combined with a microarray consisting of five 500-nucleotide probes for each virus. This enabled accurate detection and identification of West Nile and dengue viruses and was reported to have an even lower limit of detection than the routinely used RT-PCR methods. Further validation tests on human patient samples containing dengue viruses, or normal human serum spiked with YF or JE viruses confirmed that this flavivirus microarray can simultaneously screen a sample for several viruses in parallel with a high degree of confidence.

High-density resequencing microarrays (RMA) are another microarray variant for rapid detection and molecular characterization of bacteria and viruses. RMA are built from an initial set of pathogen-specific oligonucleotide probes which is then expanded through systematic sequence variation at single locations, yielding large arrays with up to thousands of sequences that are capable to detect not only the pathogens from which the sequence was derived, but also new pathogen variants that may have emerged through mutations and genetic drift. A PathogenID v2.0 RMA (PID2-RMA) that contains a set of 126 viral sequences from important pathogens, including arboviruses, was developed for the detection and genetic analysis of dengue, West Nile, and Chikungunya viruses in spiked blood samples or sera from dengue patients.[105] The test proved effective for rapid identification and genetic analysis of these three viruses, rendering this technique particularly appropriate in emergency case. Microarray analysis also enables to develop biomarkers predicting clinical outcome in dengue. One study analyzed gene-expression microarray data of peripheral blood mononuclear cells collected from DF and DHF patients during the febrile phase.[106] At early stages of dengue infection, genes involved in effector mechanisms of innate immune response were less activated in those patients who later developed DHF, whereas genes involved in apoptosis were more strongly expressed in those individuals. Such gene expression signature may contribute to proper disease management in the future as well as to a better understanding of molecular mechanisms in this disease.[107,108]

While high-throughput microarrays have proven useful for simultaneous detection of dengue virus and other arboviruses circulating in a given region,[4] they are still too expensive and complex for routine biological analysis and even more so for POC usage in resource-poor setting.[46] Other drawbacks of current microarrays include the difficulty of scaling down the array density, limited resolution of the detection scheme, significant sample concentration-dependence, and rather long hybridization time requirements.[78]

Microfluidic platforms

Microfluidic systems are also known as lab-on-a-chip (LOC) and micro total analysis systems (μTAS). They integrate, in a single chip, several functional modules for specimen processing, biochemical reactions, transportation, and product detection, with on-chip control of thermo-pneumatic pumps, micro-heaters, temperature sensors, miniaturized fluorescence detectors, sample/analyte concentrators, and filters.[109,110,111] Microfluidic techniques are increasingly incorporated into diagnostic systems due to the inherent advantages of miniaturization and integration of complex functionality. This development could move sophisticated diagnostic tools out of the developed-world laboratory and create portable POC medical diagnostics, especially in low resource setting.[72,112,113,114] The ability of microfluidic platforms to manipulate and analyze very small fluid volumes is promising for faster and more accurate diagnosis of viral infections such as dengue. Microfluidic systems for virus detection based on nucleic acid analysis or immunoassays have been developed. An automatic one-step RT-PCR diagnosis system that integrates sample purification and enrichment using superparamagnetic beads on a single chip has been reported.[115] The target virus was first captured by antibody-conjugated magnetic beads, which were then manipulated by small electromagnets made by micro-electro-mechanical systems technology. The purified and enriched virus was subjected to thermal lysis and RNA extraction, followed by RT-PCR, which were performed automatically using membrane-activated micropumps and microvalves. With this hand-held, integrated micro-RT-PCR system, two different types of viruses (DENV2 and enterovirus (EV) 71) were successfully purified, collected, and detected with a detection limit of approximately 10^2 PFU/ml. Compared with a commercial RNA extraction kit and a large-scale RT-PCR machine, the new microfluidic procedure using magnetic beads had the same

detection efficiency for the four serotypes of dengue virus.[116] A magnetic bead-based microfluidic system allowed rapid, simultaneous serological analysis of IgG and IgM associated with dengue infection.[117] Virus-coated magnetic beads were used to capture virus-specific IgM and IgG from serum samples. After on-chip magnetic purification and bead isolation, fluorescent detection antibodies were bound and the whole complexes were transported into a sample detection chamber for quantitative readout using an integrated optical module. This system integrated microfluidic control, bead purification, and optical detection into a single chip to automatically carry out the entire diagnostic process within less than 30 minutes with a sensitivity down to 21 pg virus-specific IgG. In a different work, a compact disk (CD) microfluidic device based on reciprocating flow was built to realize a rapid DNA hybridization assay for nanoliter samples.[118] This device consists of a polymer CD slab containing twelve DNA hybridization units with dengue virus DNA probes immobilized on a glass substrate. After fluorescein-labeled synthetic dengue virus target oligonucleotides were loaded, a reciprocating flow was driven by CD rotation-pause protocols, resulting in stronger hybridization signals compared to flow-through hybridization. Such CD microfluidic devices have the potential for further development into simple, portable, and possibly high-throughput platforms for rapid POC diagnostics.[119,120]

Capillary test strips or lateral flow assays can also be considered as fairly simple microfluidic systems and are the most successful POC tests to date. One direction of research and development is further simplification, while another direction is integration of more functionality, like mixing, dilution, and washing. LF devices can be reduced to their bare ingredients - patterned filter paper impregnated with reagents (the so-called bioactive paper) resulting in microfluidic paper-based analytical devices (μPADs).[121] Costs are reduced by eliminating glass and plastics; by simplified fabrication not requiring etching, vacuum pumps, and clean rooms; by independence from pumps or power; and by easily avoiding bubble formation that may cause failure in other microfluidics platforms.[122] Paper-based techniques allow performing multiple, simultaneous, and independent assays with multiplexed detection. Fast prototyping and production techniques for paper-based diagnostic devices have been proposed.[123,124] μPADs are inexpensive, easy to use, lightweight to transport, compatible with biological samples and may be handled in remote setting by non-trained personnel. An *in vitro* diagnostic device that combined RT-LAMP with a paper-based diagnostic

platform was developed for nucleic acid detection of DENV2 RNA.[125] A paper-based 96-well plate was created *via* simple wax printing, thereby forming individual test zones as reaction sites. RT-LAMP products were fluorescently quantified using fluorescent probes specific to serotype 2. The detection time of 100 minutes and a specimen volume of 2 μL for paper diagnosis render this approach highly interesting for POC application. The insights into the interactions between double-stranded DNA, fluorescent probes, and fiber structures in paper in this study are a valuable contribution to further progress in paper-based nucleic acid detection.

Despite the efforts made in developing microfluidic POC diagnostic devices, this field is still in its infancy and some issues remain, e.g. device sensitivity, multiplex analysis capabilities, assay stability/shelf life, and fabrication cost. Standardized evaluation of the performance of microfluidic versus other POC technologies in a real world setting is needed.[126,127]

Epidemiologic and clinical settings for laboratory diagnosis of dengue

In order to choose a suitable diagnostic strategy and to interpret test results appropriately, investigations focusing on the clinical value of various dengue diagnostic tests in different epidemiological and clinical settings are necessary. In endemic areas, where secondary dengue infections occur and various dengue types may be prevalent in parallel or in temporal succession, virus isolation and RT-PCR were found to be the most sensitive tests during the first 3 days of illness, followed by NS1 antigen and IgM antibody detection. After day 3 of illness, IgM antibody detection was the most sensitive method for dengue diagnosis.[128] Reduced sensitivity of the NS1 antigen in an endemic region could be due to high titers of pre-existing IgG antibodies in patients with acute secondary dengue infection.[129,130,131,132] A practical approach to dengue laboratory diagnosis in endemic countries is described in Figure 5.[128] In non-endemic regions, dengue infections occur commonly in travelers returning from endemic areas.[133,134] Dengue NS1 antigen ELISA was reported as a useful tool for confirming dengue virus infection in international travelers, especially when it

was used in combination with IgM detection.[135] NS1 rapid tests have also been proven useful in screening travelers in airports.[136]

Rapid diagnosis tests such as lateral flow tests can be used as a dengue diagnostic tool in remote or resource-limited settings. Different commercial rapid tests for detecting dengue NS1, IgM, and IgG are available but have variable sensitivity and specificity.[137] A recent study from the WHO/TDR/PDVI laboratory network showed that ELISAs for dengue detection generally performed better than the available rapid tests.[4] Therefore, results of currently available rapid tests for diagnosis of dengue must be interpreted with caution, and the individual or epidemiological consequences of a potential false negative result should be taken into account.

Dengue diagnosis in the context of patients presenting with fever

As the first clinical manifestation of dengue is nonspecific fever with a broad differential diagnosis, discriminating dengue from other febrile diseases, such as influenza, chikungunya, leptospirosis, typhoid, malaria, and rickettsia, based on the clinical presentation is a challenge, but clinically important.[138] Despite the publication of clinical guidelines, dengue can be misdiagnosed, leading to both under-diagnosis and over-diagnosis.[139] It has been reported that the febrile patients with suspected leptospirosis actually had laboratory-diagnosed dengue as the cause of their fever.[140] Conversely, some cases of suspected dengue had leptospirosis.[141,142] Co-infections of dengue occurring with leptospirosis,[143] malaria,[144,145] typhoid,[146] and chikungunya[147] are well known, further adding to the diagnostic challenge. For workup of febrile patients, particularly in the tropics and sub-tropics where acute fever or acute febrile illness (AFI) is common, a multidimensional diagnostic approach may be more appropriate than testing for a single infection using a single parameter.[42,43,148] Such testing will take multiple infectious agents and multiple diagnostic parameters per microorganism into account for therapeutic decision making in febrile patients. Ideally, the diagnostic parameters selected into a multiplexed panel should include the biomarkers not only for pathogen identification, but also for prediction of disease severity and prognosis. For example, early prediction of

severe dengue is very important for the care of dengue patients. But currently there is no reliable clinical or laboratory means to accurately predict the severity of individual cases. Numerous studies have focused on identifying biomarkers in clinical samples that differ between severe and non-severe cases. Some laboratory parameters such as viral load, cytokines and cytokine receptors are promising to be developed as early biomarkers of severe dengue.[149]

Availability of multiplexed tests that can quickly identify the causative pathogen, predict the progression and exclude alternative infectious agents is highly desirable from the standpoint of an individual patient but may also have an important impact on public health.[42] Recent advances in diagnostic technology that allow detection of multiple targets in a specimen include advanced multiplex nucleic acid tests, array based immunoassays and bead/flow-based assays.[50,150,151,152] Such new diagnostics have considerable potential to improve care, target treatment, and reduce the cost of unnecessary prescriptions and the downstream effects of antimicrobial resistance.[153,154] Unfortunately, these diagnostic tools are often unavailable in developing countries due to cost and infrastructure issues.[44,155] In such resource-limited settings, availability of POCT is particularly important, as the only alternative may be no diagnostic support at all.[150] While single-infection POCTs are increasingly used for tropical infections, there is a large unmet need for commercial multiplexed POCT that can be used in resource-limited settings for the detection of multiple etiologies of AFI. Currently, technology development projects (such as EU FP7 DiscoGnosis project)[156] explore multiplexed microfluidic immunoassays and LAMP tests for detecting multiple infective agents at the point of care in a single assay. Certainly, such tests will need thorough clinical validation in relevant epidemiological settings before broad application. However, we expect that multiplexed POCT for workup of fever in developing countries will eventually replace the current syndromic approach and bring a benefit to the individual patient by early and correct treatment while simultaneously relieving healthcare systems from the cost and side-effects of unwarranted therapies.[157,158]

Multi-parameter assays might show that clinical reality in individual patients may be more complex than often assumed because multiple infections may be present, infection parameters change over time, and pretest probability (e.g. epidemiologic setting) may influence the interpretation of multiparameter tests. For

clinical decision making, this complexity needs to be reduced to simple clinical management strategies, but we cannot expect that this expertise is available to healthcare workers at all education levels. This challenge is an opportunity to develop point-of-care devices that incorporate advanced algorithms which take patient characteristics, epidemiologic setting, and multiple test parameters into account but have a very simple user interface and possibly an explicit management recommendation.

Challenges and future needs for dengue diagnostic tests

During the last decade the diagnostic tests of dengue infection have been greatly improved. However, due to the uniqueness of the dengue virus and the spectrum of disease resulting from infection, some issues still warrant further investigation (Table 2).

The continued development of sensitive, simple, rapid, inexpensive, less cross-reactivity and serotype-specific tests that allow for early dengue diagnosis are still required. Also, during developing an assay the wide geographic serotype and genotype variability needs to be taken into account. The complicated pathogenesis and clinical features of dengue make it difficult to create a single assay to confirm dengue infection. A combination of current clinical diagnostic tests (multiple tests) would improve sensitivity.[70] Many efforts are being made to develop multiplexed POC tests (such as microfluidic diagnostic device) covering multiple infectious agents and multiple parameters per pathogen to discriminate dengue from other flaviviruses and/or other tropical infectious diseases.

The majority of dengue cases are uncomplicated and will be self-limiting; however, a small proportion (5-10%) progress to severe dengue, which is characterized by hemorrhage and shock and can cause death if it is not managed appropriately.[24] Early detection of shock and other complications and the immediate replacement of fluid have been shown to reduce the mortality, and therefore it is important to develop prognostic indicators to predict the severity of disease, allowing for more efficient patient triage and treatment. The previously established warning signs and coexisting conditions, as is recommended by the

WHO in 2009 dengue guidelines, may be used as early predictors for severe dengue.[4] Current research has centered on using protein and nucleic acid detection technologies on patient blood specimens to discover and identify early-onset biomarker/biomarker panel that reliably predict the progress into severe manifestation.[9] Moreover, because secondary infection with another dengue serotype increases the risk of developing more severe forms of the diseases,[31] developing assays to accurately differentiate primary and secondary dengue infection will assist in predicting the severity of disease and could permit for better patient management.

New miniaturized devices and technologies (such as biosensor-based assays and microfluidic diagnostic device) for dengue diagnosis have high potential to solve drawbacks of current technologies. But validating such tests in different regions and assessing them to the needs of that region are urgently needed.[70] In addition, for exploiting POC devices used in tropical and developing countries, climatic condition for use/storage, shelf life and cost per test need to be considered.

Conclusion

Dengue is a growing healthcare concern in developing and developed countries. Conventional laboratory diagnostic tools for dengue infection require sophisticated equipment and well-trained personnel, and thus do not meet all ASSURED requirements for successful POC diagnosis in resource-limited settings.

Improved rapid and miniaturized POC tests for dengue diagnosis are needed for various epidemiologic contexts. The currently available lateral flow or immunochromatographic strip tests are valuable complements of conventional analytical techniques for simple rapid dengue detection in both endemic and non-endemic areas, but their sensitivity remains to be further improved. Advances in bioelectronics and micro- and nanofabrication technologies allow development of new miniaturized POC devices and analytical methods, which may complement or replace conventional laboratory assays for rapid and accurate detection of dengue infection. Recent progresses, focusing on three label-free biosensor methods and two miniaturized platforms for dengue are discussed in this review. Devices based on such tests hold great potential for POC diagnosis but are still in the early stages of development. Before a new dengue POC diagnostic device is translated into clinical use, thorough performance and quality evaluation in well-characterized dengue and other fever sera panels, as well as field testing, including multicenter testing in specific epidemiologic contexts, is required. Technical requirements including climatic conditions for use/storage, shelf life, and cost per test are paramount for their application in tropical and developing countries. New test modalities for dengue need to combine sensitivity, specificity, time consumption, as well as low cost (below 1$ / test), and also are required to prove their value in the context of a pathogen that not only occurs in several serotypes but also displays genetic and antigenic drift over time. To diagnose fever, the integration of dengue detection in comprehensive multiparameter POC tests is desirable. Application of such multi-parameter tests in the diagnostic workup of febrile patients will require translation to the field, new fever workup strategies, and training of healthcare workers, and may profit from advanced integrated decision-making tools that incorporate smart algorithms to deliver simple answers to the end user.

Figure legends

Figure 1. **Historical perspective on diagnostic technologies for dengue**

Figure 2. **The Kinetics of dengue virus, NS1 antigen, and IgM/IgG antibodies during a primary or secondary infection**[11,66]

Figure 3. **Diagram of a typical Immunochromatographic strip (ICS) for microbial detection (a, A schematic of the ICS assay format; b, A test example)**: in this ICS test, bacteria could be replaced with dengue antigen

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Figure 4. **Schematic of the dengue virus diagnosis process using surface plasmon resonance biosensor** [link]

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Figure 5. **Algorithm for laboratory diagnosis of clinically suspected dengue cases in endemic setting** [link]

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Table

Table 1. **Conventional and emerging laboratory diagnostic methods for dengue infection**

Methods/Technologies		Advantages	Disadvantages	Major applications / markers
Conventional laboratory diagnostic methods[11,66,75]				
Virus detection:	**Virus culture**	• Confirmation test; • Specific; • Identifies serotypes.	• Requires high-level equipment, technical skills, manpower, and time consuming (several days); • Expensive.	Detection of dengue pathogen
Nucleic acid amplification:	**RT-PCR (Real-time RT-PCR, NASBA, RT-LAMP, etc.)**	• Confirmation tests; • Fast (Results in 24-48 hours); • Sensitive (80-90%) and specific (>95%); • Serotype discriminating.	• Easy sample contamination; • High technological demands.	Dengue viral RNA detection
Serological assays:	**ELISA**	• Easy to perform; • Fast (Results in 4-6 hours); • Less expensive than virus isolation or RNA detection.	• Requires equipment (microplate reader); • False-positive due to cross-reactivity.	Dengue NS1, IgG or IgM detection
Clinical rapid diagnostic tests[11,75]				
Point-of-care tests:	**Lateral flow test**	• Less time of analysis (15-20 min); • Simple, user friendly and easy to perform; • Require no extra equipment or supplies; • Less expensive than virus isolation or RNA detection.	• Mostly qualitative or semi-quantitative; • False-positive due to cross-reactivity; • Less sensitivity and specificity than ELISA-based tests; • Variable sensitivity and specificity among different commercial rapid tests.	Dengue NS1, IgG or IgM detection
Emerging diagnostic tools				
Label-free biosensors:	**Quartz crystal microbalance (QCM)**[81,83]	• Label free; • Easy-of-use; • Economy; • Better sensitivity; • Relatively simple technology in its production; • Potential for POC diagnosis.	• Prone to serum proteins interference; • Issue of robustness should be improved.	Detecting dengue viral protein (NS1, E protein);[82,83,84] viral genome[85]
	Surface plasmon resonance (SPR)[89,92]	• Label free; • Real-time monitoring; • Low sample consumption; • High throughput; • Remarkable sensitivity; • Quantitative.	• Bulky nature of the detection apparatus.	Detecting dengue viral antibody (IgM);[88,90] viral protein (NS1)[91]
	Electrochemical impedance spectroscopy (EIS)[93,98]	• Label free; • Simple; • Cost effective; • Quantitative; • Ease of miniaturization; • Provides time-dependent information.	• Time-consuming technique; • Moderate sensitivity.	Detecting dengue virus particles;[98,100] dengue oligonucleotide[101]
Microarrays[46,78]		• Extremely high throughput; • High sensitivity.	• High running cost; • Requires expensive equipment and high technical skills; • Not suitable for routine and POC usage; • Time consuming (several days).	Detection and genetic analysis of dengue;[104,105] To develop dengue prognostic biomarkers based on gene expression signature[106,107,108]
Microfluidic platforms[72,126,127]		• Miniaturization, increasing portability and disposability, suitable for POC usage; • Low reagents cost, less waste, less	• Novel technology and therefore not yet fully developed; • Lack validation and	Detecting dengue viral RNA;[115,116] viral antibody (IgM & IgG)[117]

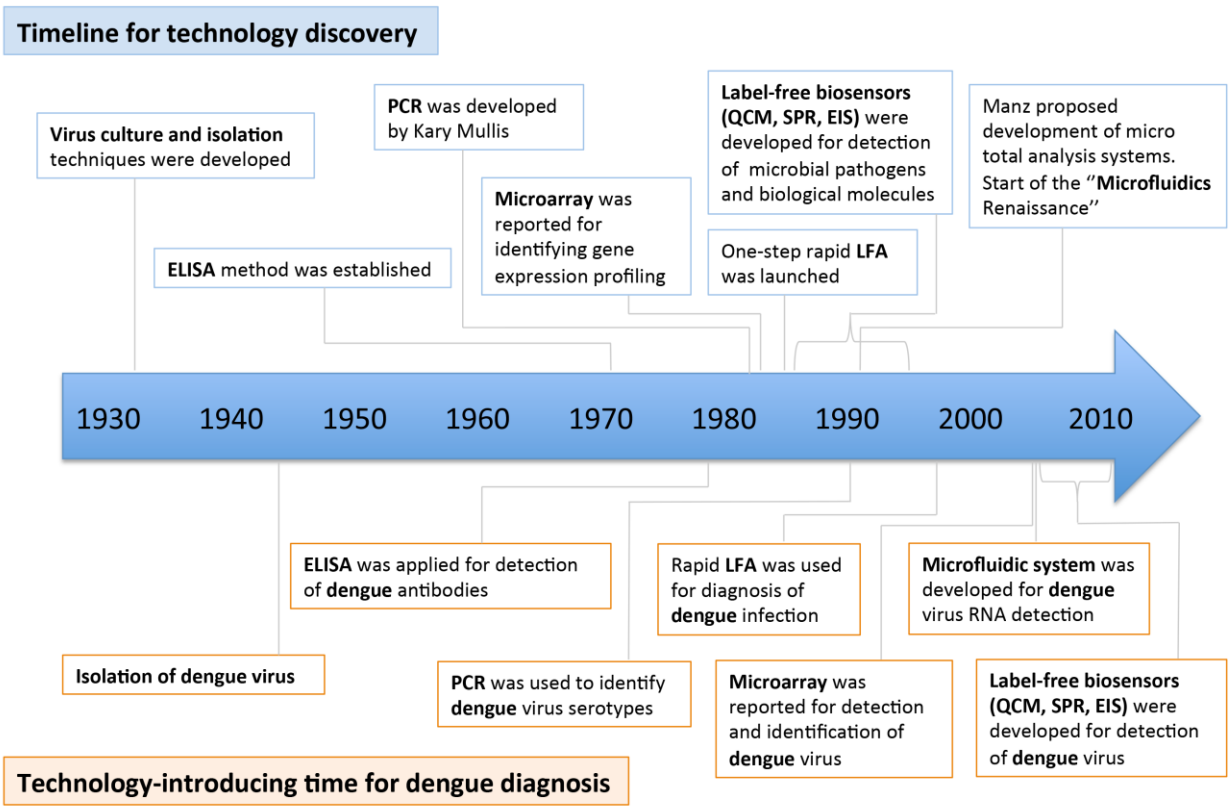
	required sample volumes; • Faster analysis and response times; • Better process control; • Capability for parallel operation and multiplex capability; • Minimized handling of hazardous material.	standardization; • Complex and somewhat expensive fabrication procedures.

RT-PCR: reverse transcription-polymerase chain reaction; NASBA: nucleic acid sequence-based amplification; RT-LAMP: reverse transcription-loop-mediated isothermal amplification; ELISA: enzyme-linked immunosorbent assay; POC: point-of-care.

Table 2. **Challenges and future needs for dengue diagnostic tests**

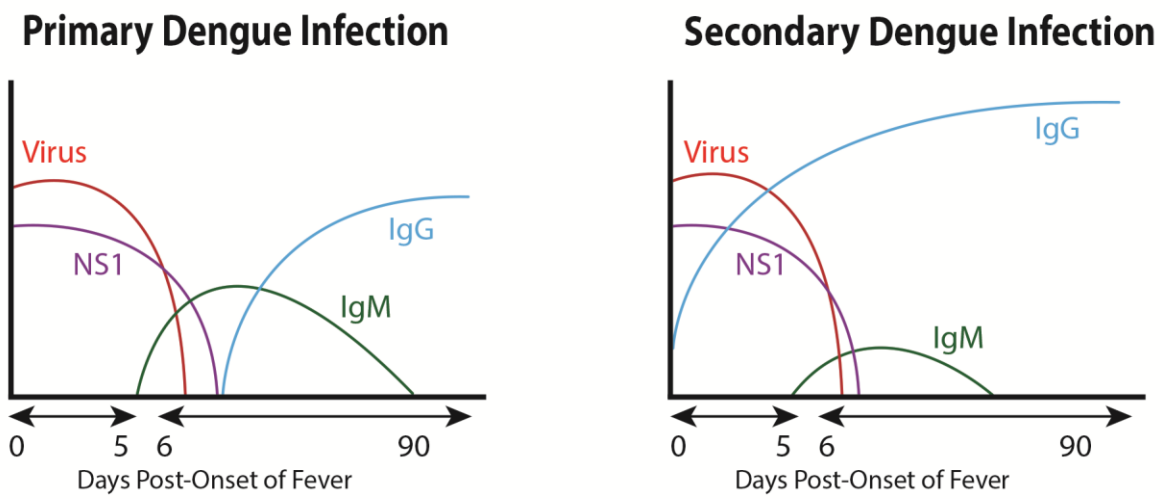
Challenges/issues	Future needs/directions
• Discriminating dengue from other flaviviruses and/or other tropical infectious diseases	• Further improving dengue diagnostic techniques to avoid cross-reactivity; • Developing multiplexed POC tests (such as microfluidic diagnostic device) covering multiple infectious agents and multiple parameters per pathogen.
• Predicting the likelihood of a dengue patient to develop a severe form of the diseases	• Discovering and identifying early-onset biomarker/biomarker panel of disease severity; • Accurately differentiating primary and secondary dengue infection.
• Evaluating new miniaturized devices and technologies (such as biosensor-based assays and microfluidic diagnostic device) for dengue diagnosis	• Establishing standardized approach to validate such tests in different regions and assess them to the needs of that region.
• Exploiting POC devices used in tropical and developing countries	• Climatic condition for use/storage, shelf life and cost per test need to be considered.

POC: point-of-care.



Abbreviations: ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; QCM, quartz crystal microbalance; SPR, surface plasmon resonance; EIS, electrochemical impedance spectroscopy; LFA, lateral flow assay.

Figure 1



Main clinical diagnostic tests: Virus---virus culture, RT-PCR (reverse transcription-polymerase chain reaction); NS1, IgG, IgM---ELISA (enzyme-linked immunosorbent assay), lateral flow assay.

Figure 2

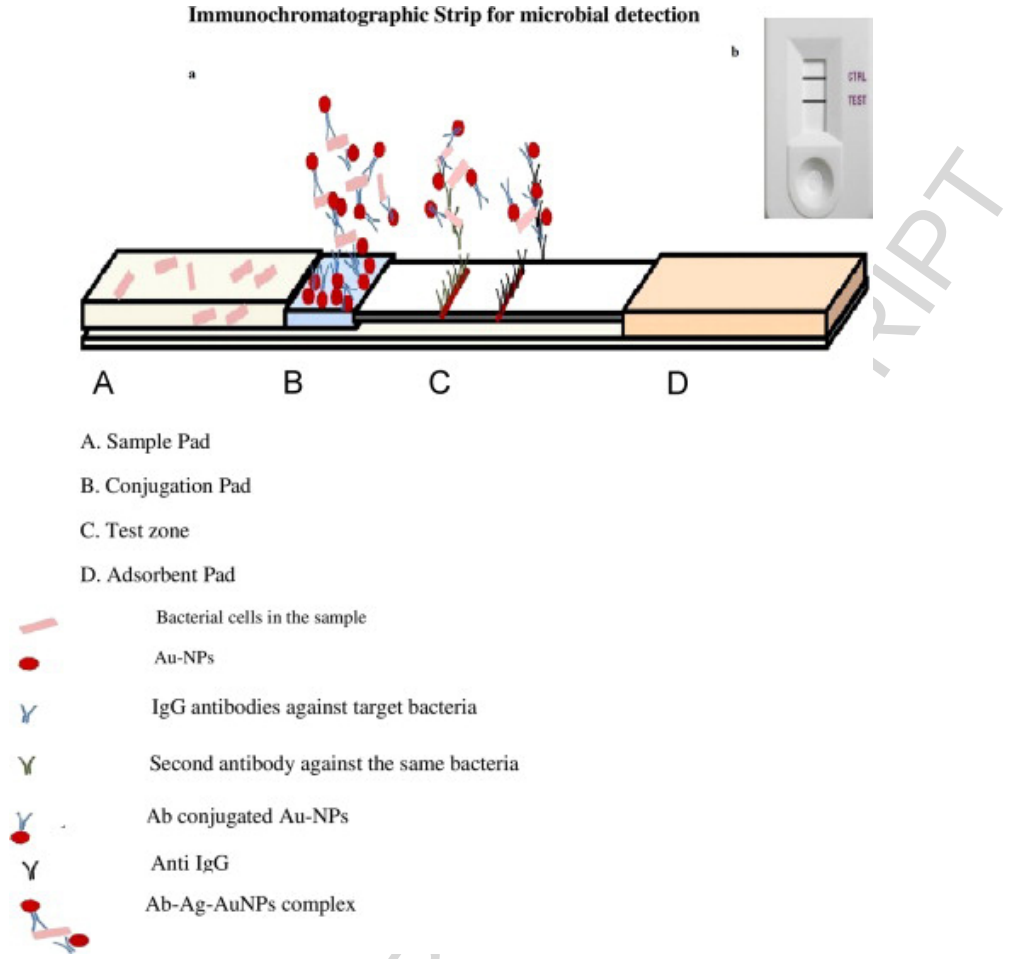


Figure 3

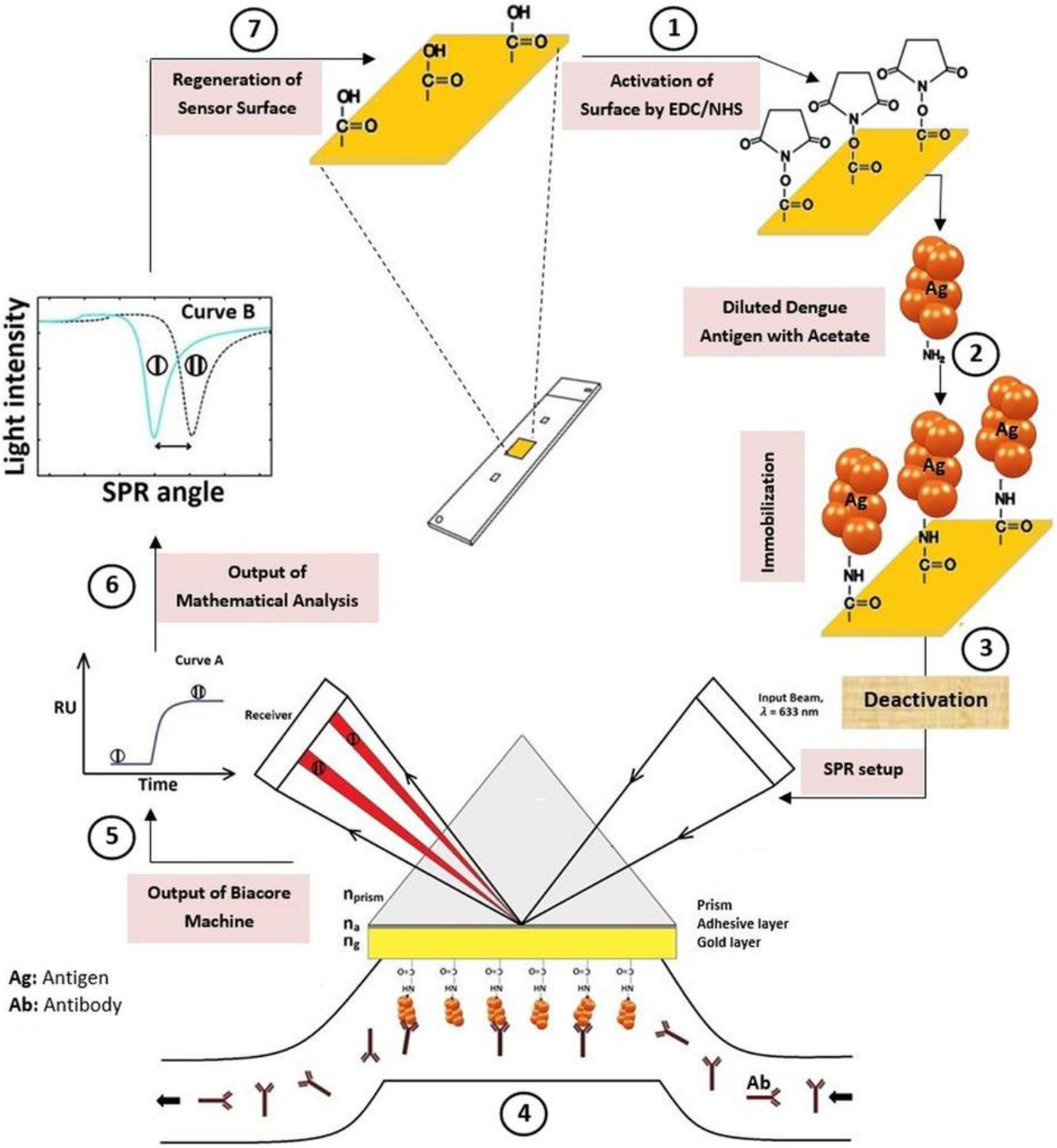


Figure 4

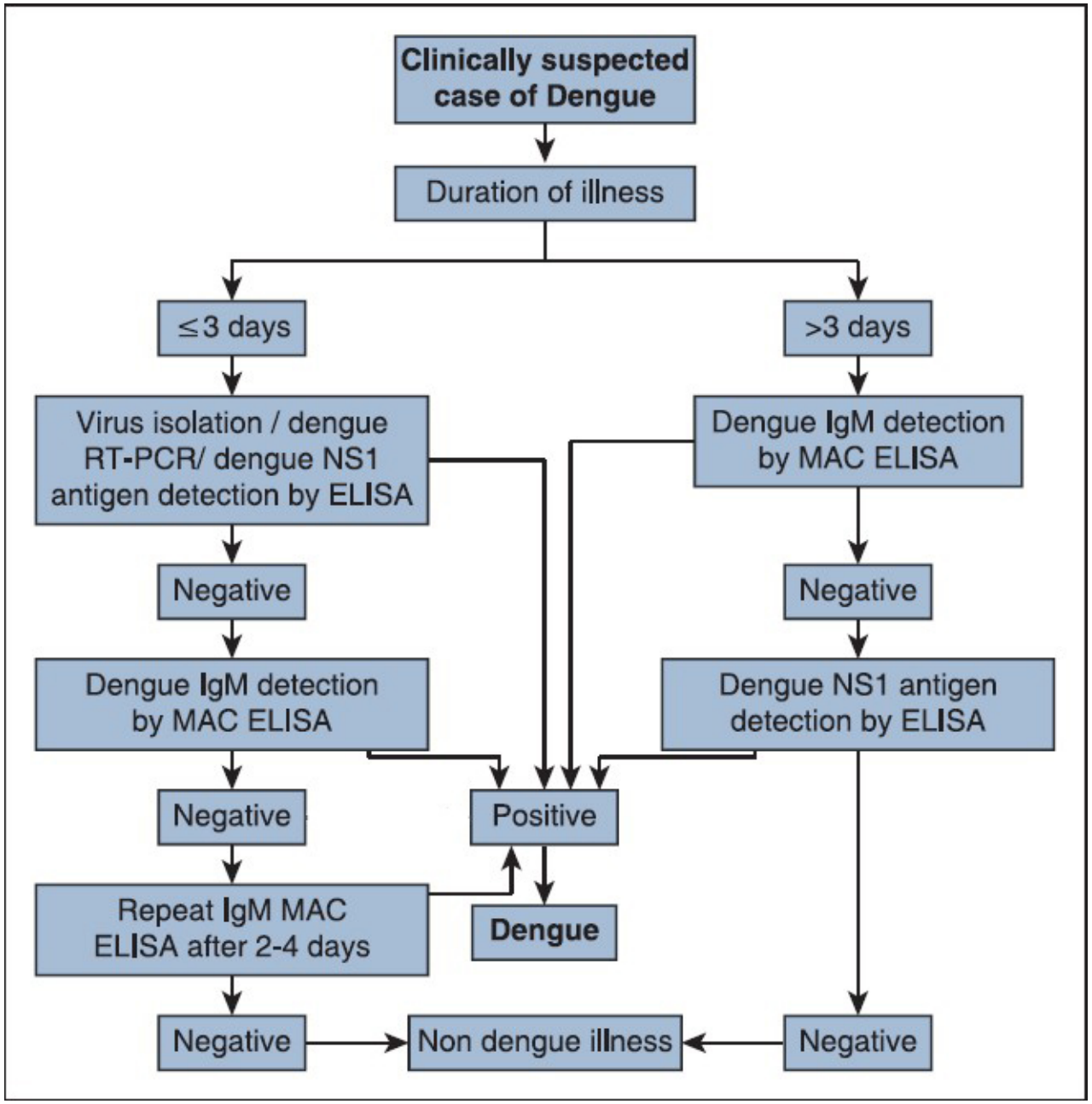
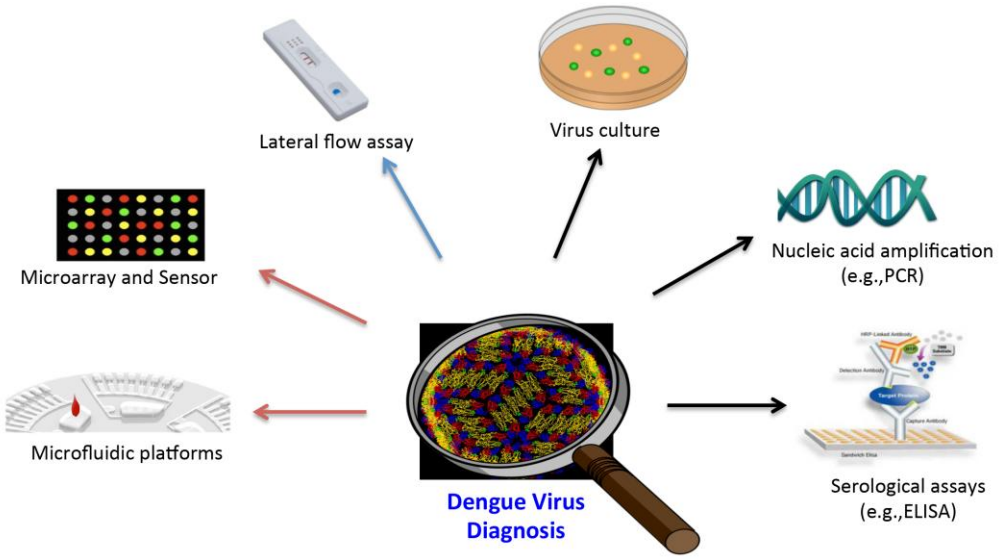


Figure 5



Graphical abstract text

Dengue is an arthropod-borne viral disease that is mostly endemic in tropical and subtropical areas. Rapid diagnosis of dengue infection is critical to limit pathogen spread and alleviate morbidity and mortality. Recently, the growing capabilities of the micro/nanotechnologies have brought new diagnostic options for this infectious disease. This review presents the challenges, the advances and the clinical implications of current and novel diagnostic technologies for dengue infection, especially emphasizing rapid and micro/nanotechnologies-based diagnostic tools, including label-free biosensor methods, microarray and microfluidic platforms.