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New Tools for Dengue Diagnostics

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Abstract

Dengue caused by four antigenically distinct serotype remains a serious health concern around the world, particularly in the tropical areas. Clinical signs and symptoms of this disease are indistinguishable from other infectious disease; therefore, laboratory diagnosis is very crucial for confirming the disease that will be useful for the patient's management. In laboratory, dengue can be confirmed using cell culture, RNA detection, and serological detection based on ELISA and immunochromatographic test. However, each of these methods has certain practical limitations. Therefore, researchers from all over the world have been working to address these limitations. In this chapter, we will highlight the current research toward the development of novel point-of-care test for the diagnosis of dengue in acute and convalescent phase.

Keywords: dengue, diagnosis, microfluidic, RT-LAMP, biosensor

1. Introduction

Despite the significant advancement in medical sciences, dengue remains a serious public health concern in more than 100 countries, precisely in tropical and subtropical parts of the world. Dengue has alarming situation in Southeast Asia, South America, and Africa. Approximately, half of the world's population living in dengue endemic area is at the risk of getting dengue infection. Evidence shows that every year about 390 million dengue infection appears worldwide, of which 100 million cases are found to be symptomatic and require medical attention [1–3].

Clinical symptoms typically appear 4–7 days following the mosquito bite and may persist for 3–10 days. Clinical manifestation of dengue varies from asymptomatic to acute febrile illness with headache, vomiting, severe myalgia, rash, retro-orbital pain, and arthralgia [4–6]. Classically, dengue was categorized as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Nevertheless, classification was revised by the WHO in 2009 and classified it as dengue with or without warning signs. This version of classification divides dengue into three clinical phases: febrile, critical, and recovery phases [7, 8]. In the febrile phase, patient develops high fever due to acute viremia, and this phase lasts for 2–7 days. Critical phase usually lasts for 2 days and is indicated by plasma leakage, hemorrhage, and low platelet number. If patients survive in critical phase, then they will recover from the disease at the third phase known as recovery/convalescent phase [9, 10].

The spherical and enveloped dengue virus (DENV) which belongs to the family *Flaviviridae* is the causative agent of dengue. This virus is 50 nm in diameter and contains about 11 kb positive-sense single-stranded RNA genome that codes for three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins [11, 12]. The DENV is transmitted to human by the bite of *Aedes aegypti* and *Aedes albopictus* mosquito which usually breed in the clean water in urban areas [13, 14]. Each serotype of DENV can cause dengue and trigger inimitable immune response in host which provides long-term immunity to that particular serotype but limited and partial immunity against three other serotypes [15, 16]. Although, the four DENV serotypes are antigenically different but genetically are identical as they share about 65% of their RNA sequences [17, 18].

2. Diagnosis of dengue

At this time, a tetravalent dengue vaccine has been developed but due to its lack of protection on non-exposed individuals, it is not an effective option. On the other hand, antiviral drugs for curing the dengue are not available; thus, accurate and timely diagnosis is of utmost importance for appropriate management of a patient suffering from severe dengue [19]. However, diagnosis of dengue based on clinical manifestation is quite complicated as the signs of dengue are very common in other febrile illness; therefore, diagnosis is very challenging using this approach [10, 20]. Thus, laboratory confirmation is needed for definite diagnosis. Laboratory confirmation of dengue can be obtained using several techniques such as virus isolation, polymerase chain reaction (PCR)-based detection of viral genome, nonstructural protein 1 (NS1) antigen detection, and serological detection of dengue-specific antibodies such as IgM and IgG [21]. Nevertheless, effective application of each diagnostic technique depends on the disease stages.

2.1. Recent advancement in the diagnosis of dengue

The WHO set "affordable, sensitive, specific, user-friendly, rapid, easy to handle and deliver to those who need them (ASSURED)" as the characteristics of an ideal point-of-care test for resource-limited countries [22, 23]. With the advancement of science and technology, several

novel diagnostic platforms emerged for the diagnostic of infectious disease such as biosensor, microfluidic, loop-mediated isothermal amplification (LAMP), and so on. These diagnostic platforms were also explored by the researchers for developing a point-of-care test for the diagnosis of dengue. The below section will briefly give an insight into some of these platforms investigated for the diagnosis of dengue.

2.1.1. Biosensor platform

The criteria of point-of-care test resulted attractive for industry and researchers in order to develop and satisfy the qualification of ideal diagnostic test. Therefore, numerous researchers around the world have been working on biosensors in a search of potential point-of-care test as they offer several advantages such as high sensitivity and specificity, simple instrumentation, rapid assay outcome, portability and disposability of developed tools. Several researchers have reported biosensor for the diagnosis of dengue. In this section, we take a glance over current biosensor methods aimed to improve the diagnostic of dengue using different biomarkers.

2.1.1.1. NS1 antigen detection

Omar et al. reported an electrochemical immunosensor based on screen-printed carbon electrodes (SPCE) for the detection of dengue NS1 antigen. Anti-NS1-capturing antibodies were immobilized on BSA-modified working electrode of SPCE. The detection was based on the measurement of electron transfer resistance before and after the NS1 binding. The study demonstrated that the immunosensor successfully detected NS1 antigen with a limit of detection (LOD) of 0.3 ng/mL and the linear range was 1–200 ng/mL [24]. Pirich et al. worked on piezoelectric immunosensor to detect dengue NS1 antigen. For the enhanced binding of anti-NS1 antibodies, sensor surface was coated with a thin film of bacterial cellulose nanocrystals. The formation of antigen antibody complex was then analyzed using quartz crystal microbalance. The study showed that immunochip was able to detect dengue NS1 antigen from serum with a LOD of 0.32 μg/mL [25]. In another study, NS1 was detected using Langmuir–Blodgett and gold nanoparticles (AuNP) composite as a biosensing surface. The anti-NS1 antibodies were immobilized on the biosensing surface and then the sample containing NS1 antigen was pipetted on it. Subsequently, the biorecognition event between anti-NS1 antibody and NS1 antigen was detected through electrochemical impedance spectroscopy. The study showed that developed electrochemical immunosensor was capable to detect dengue with a LOD of 1.19 ng/mL in spiked serum sample [26].

2.1.1.2. Detection of dengue IgM antibodies

Jahanshahi et al. targeted the dengue IgM antibody as an analyte in an optical biosensor. In the assay, four serotypes of DENV were used as ligands for capturing IgM antibodies. The assay time of optical biosensor was just 10 minutes and required a minimum volume of 1 μl of sample to perform it. The sensitivity of developed sensor was ranging from 83 to 93% and specificity was 100% [27]. Ortega et al. reported a novel "Magnetic Paper-Based ELISA" for

isotype IgM-dengue antibodies detection. In the assay, magnetite nanoparticles were deposited on cellulose paper sheet. The antihuman IgM-capturing antibodies were immobilized on nanoparticle using a cross-linker, namely polydopamine. The concentration of IgM antibodies captured by antihuman IgM antibodies was determined using Bradford assay and SDS gel electrophoresis. The study claimed to have 700 times lower LOD than conventional ELISA [28]. Ortega et al. reported an electrochemical immunoassay using lead sulfide quantum dots as detection label for enhancing the sensitivity of assay by conjugating with anti-IgM detection antibodies. The immunoassay was performed on ELISA microplate, and electrochemical response generated by acid dissolution of detection label was measured on SPCE. The differential pulse anodic stripping voltammetry was used to measure the electrochemical response. Using this technique, immunosensor successfully detected dengue IgM antibodies with a LOD of 130 ng [29]. Wong developed an immunosensor using long-range surface plasmon-polariton waveguides for the detection of dengue IgM antibodies. The developed immunosensor detected IgM antibodies from a serum sample with a LOD of ~22 pg/mm^2 [30]. Liu et al. fabricated a label-free immunosensor based on optical fiber long-period grating (LPG) for detecting dengue IgM antibodies. For the detection of IgM antibodies, the LPG was modified with a film of three layers containing poly(allylamine hydrochloride) and silica nanoparticles. Later, anti-IgM antibodies were immobilized via covalent binding on modified surface. Subsequently, when the sample containing analyte IgM antibodies was added, it caused an increase in refractive index of film coated on the surface of LPG leading to a wavelength shift. This wavelength shift suggested was due to the binding of IgM antibodies with anti-IgM antibodies. The optical biosensor developed in this study detected IgM antibodies with a detection limit of 15 pg/mm^2 [31].

2.1.1.3. Detection of dengue genome

On the other hand, some researchers focused on viral genome as a target analyte in the hunt of a point-of-care test. Chan et al. developed a genosensor using a silver nanocluster (Ag NC) as hybridization indicator of target DNA with probe DNA. In the assay, the target DNA was synthesized from dengue RNA extracted from a mosquito. Afterward, the target DNA was hybridized with dengue probe DNA and amplified using isothermal amplification. Following the amplification, the product was mixed with $AgNO_3$. Subsequently, the reduction agent $NABH_4$ was added to mixture to produce Ag NC which was observed using a UV light. This study showed that the developed genosensor could detect DENV genome with a LOD of 100 nM [32]. Jin et al. developed an electrochemical genosensor by modifying graphene oxide surface with SiO_2 particles for enhancing the electrochemical properties of the surface. The negatively charged oligonucleotide primer specific for dengue complementary DNA was immobilized on the graphene-oxide-modified surface. Afterward, the sample containing target complementary DNA was pipetted. It was found that the oligonucleotide primer hybridized with complimentary DNA indicating the presence of dengue DNA in the sample. EIS was carried out on three electrode cell using PBS solution, and impedance spectra were measured. The study claimed to detect dengue DNA with a LOD of 1 femto-molar concentration of viral genome [33].

Another genosensor was developed using silicon nanowire to carry out electrochemical-based assay. First of all, silicon nanowire surface was pretreated for immobilizing the probe DNA. Later, the target DNA was added on the surface. The hybridization between probe and target DNA was detected through variation in current, conductance, and resistance on genosensor because of negative charges which increased with the binding of probe and target DNA. The genosensor developed in this study successfully detected DNA with a LOD of 2.0 fM [34]. Ariffin et al. employed nickel(II) salphen complex as DNA hybridization indicator for developing optical genosensor to detect DENV DNA. The indicator was known as a DNA intercalating agent. Porous silica nanospheres (PSiNs) were fixed on round plastic supporting case as carrier matrix. PSiNs were pretreated with glutaric acid followed by immobilization of DNA probe. Subsequently, complementary DNA analyte intercalated with nickel (II) sal-phen complex was pipetted on PSiNs' surface. The fiber optic reflectance spectrophotometer was used to measure the reflectance intensity of hybridization of target DNA with probe DNA. The study demonstrated that genosensor was capable to detect dengue DNA with a LOD of 0.2 aM [35].

Multicolor triangular silver nanoparticles (TAgs) were used by Vinayagam et al. as detection labels for the detection of DENV RNA. TAgs were conjugated with capture probe and reporter probe which will hybridize with different regions of target RNA. Later, these capture and reporter probes were pooled with analyte RNA, and hybridization was promoted using NaCl. The reaction was left for 10 minutes at 37°C. In comparison to non-hybridization, the hybridization of probes with target RNA was more stable in the presence of NaCl and, thus, develops colors and determines the presence of dengue RNA [36]. Tripathy et al. reported a genosensor was based on Manganese (III) Oxide (Mn_2O_3) nanofibers modified glassy carbon electrode (GCE). The COOH group was introduced on the surface and activated via EDC/NHS cross-linker. The capture probe was then immobilized via NH2 group with COOH group on GCE. Subsequently, the sample containing target DNA was added, and electrochemical responses were measured against a standard calomel reference electrode. The study revealed that nanoscale genosensor successfully detected dengue DNA and LOD of the sensor was found to be 120 zeptomoles [37]. Rashid et al. worked on electrochemical genosensor that is developed on screen-printed gold electrode modified with silicon nanowires (SiNWs). SiNWs were deposited on SPGE using various chemical pretreatment. Following the deposition of SiNWs, the thiolate DNA probe were immobilized on sensing surface. Subsequently, the sample containing target DNA was pipetted to hybridize with probe DNA. The electrochemical response was measured in the presence of redox indicator using CV and DPV. It was found that the developed biosensor was able to detect dengue DNA with a LOD of 1.63×10^{-12} M [38].

2.1.2. RT-LAMP platform

Loop-mediated amplification (LAMP) is a modified version of PCR in which a uniform temperature ranging from 60 to 65°C is applied using a water bath for nucleic acid amplification and, thus, obviates the requirement of a thermocycler [39, 40]. This approach to nucleic acid amplification relies on strand displacement reaction that produces a self-complementary single-stranded loop structure for binding the primers and amplifies target with high specificity

and rapidity [41, 42]. The resulting DNA product can be observed by measuring the turbidity produced from the precipitation of by-product magnesium pyrophosphate [43, 44]. In addition, the amplification product can be visualized using UV in the presence of fluorescent dyes which intercalate in double-strand DNA. The target DNA can be visualized either by the naked eyes or quantified by turbidimeter. In comparison to conventional PCR, LAMP is highly specific as it employs 4/6 primers that specifically recognize 6/8 distinct sequence of target DNA [45, 46]. Sahni et al. developed and evaluated RT-LAMP assay for the detection of dengue. In this study, 279 samples including 100 dengue positive, 100 dengue negative, and 79 samples from healthy person for negative control were evaluated for testing the sensitivity and specificity of the developed RT-LAMP assay and compared with conventional RT-PCR. The study showed that RT-PCR detected dengue in 77 samples while RT-LAMP showed good sensitivity and detected dengue in 83 samples. The diagnostic specificity analysis revealed the developed assay did not show any cross-reactivity [47]. In another study, a different approach was used to design dengue-specific primers for developing RT-LAMP assay. This study exploited a highly conserved dengue NS1 gene which study claimed to have >90% sequence similarity among different genotypes within each serotype. The assay was single step and carried out in four tubes, each one was specific for distinct dengue serotype. For the visualization of RT-LAMP product, Genie® II fluorometer was employed for real-time fluorescence detection. The sensitivity of RT-LAMP PCR was compared with CDC 1-4 real-time PCR. The study showed that the developed RT-LAMP was equally effective in discriminating dengue in the acute phase. The limit of detection of this nucleic acid amplifier was found to be 100 copies of viral RNA extracted from each serotype. The specificity of RT-LAMP evaluated using the RNA of four closely related *Flavivirus* is found to be very selective [48]. Dauner et al. developed pan-serotype dengue RT-LAMP and investigated the sensitivity of RT-LAMP using a panel of clinical samples confirmed by qRT-PCR. The study showed that pan-serotype RT-LAMP could discriminate dengue with a sensitivity of 86%. This study also visualized the amplification of target gene on lateral flow assay [49]. In one study Lau et al. employed hydroxynaphthol blue (HNB) dye for the colorimetric visualization of RT-LAMP-amplified product through the naked eye, thus, making the assay simpler as it does not require specific tool to interpret the LAMP product. The assay was developed in a single tube as multiplex RT-LAMP to detect any of the dengue serotype present in the sample. Serotype-specific primers were developed using 3′ noncoding region gene sequences for DENV 1–4. The study revealed that RT-LAMP was able to discriminate all four dengue serotypes with high sensitivity and short assay time of 45 minutes [50]. Hu et al. reported a RT-LAMP technique which employed primers designed from 3′UTR, a highly conserved gene in dengue. The 3′UTR sequence of dengue genome was obtained from GenBank, and its multiple sequence alignment was achieved using Clustal X. 2.0. In this study, RT-LAMP evaluated using mixture of clinical and reference samples containing all four serotypes of dengue. It was demonstrated that multiplexed RT-LAMP detected all the four serotypes and showed high sensitivity as this method was able to detect as low as 10 copies of viral RNA. In addition, virus found to be selective as it did not amplify the RNA of any closely related *Flavivirus* when tested with developed multiplexed RT-LAMP [51]. Lopez et al. developed RT-LAMP using a novel approach for designing LAMP primers that aimed to match the sequence of all circulating dengue virus throughout the world. This study used fast-growing sequence

databases having 932 entries of complete dengue genome sequence, and, thus, covered a huge diversity reported at that time. A mixture of four final reaction primers was achieved using a blend principal component analysis of the full dengue virus genome, and LAMP primers were designed through LAVA software. The developed RT-LAMP is found to be selective and did not cross-react with other types of *Flavivirus*. The assay for DENV1 and DENV2 were validated using blood and serum samples obtained from different regions [40].

2.1.3. Microfluidic platform

Lab-on-chip (LoC) technology allows the real-time detection of target analyte by integrating multiple laboratory process (such as biological sample preparation, processing, and analyzing) on a microprocessor chip into a completely automated and controlled analytical device [52–54]. Usually, LoC exploit the microfluidic platform owing to its ability to handle very small volume of bodily fluid, less than pico-liters, in micrometer scale channels with dimensions of tens to hundreds of micrometers [55, 56]. In addition, LoC based on microfluidics offers several other advantages such as miniaturization, short assay time, portability, user-friendly, and amenable for multiple detections of target analytes [57, 58]. Since 2000, this technology has been widely used in the field of diagnosis for developing a point-of-care test [59]. Weng et al. incorporated microfluidic platform with ELISA assay for reducing the detection time of the assay and lowering the sample volume and developed suction type microfluidic immunosensing chip for the identification of DENV. Microfluidic chip designed in this study contained a multifunctional micro-transport unit for the transportation and mixing of reagents. To reduce nonspecific protein binding polydimethylsiloxane, the material used for the fabrication of microfluidic surface was modified. The study demonstrated that microfluidic-based immunochip was able to detect dengue using a small volume of 12 μL of dengue sample. The developed assay demonstrated high sensitivity with a LOD of 10^3 PFU/mL and short assay time of half an hour and makes more rapid test than ELISA which usually takes 3–4 hours for detection [60]. Hosseini et al. develop a hybrid platform by combining microsphere and microfluidic disk for the detection of dengue virus. Microspheres were selected as they offer large specific surface area, and microspheres designed with functional group were integrated into microfluidic disk to promote biorecognition event. For the maximum utilization of microspheres' specific surface area for bimolecular interaction, micromixing system was fitted in microfluidic disk. The detection principle of this assay was based on sandwich ELISA technique. Utilization of this hybrid platform reduced the long incubation period from several hours to 5 minutes and demonstrated high sensitivity by detecting as low as few units of dengue virus [61]. Aeinehvand et al. employed centrifugal microfluidic platforms for the detection of DENV. Microballoon mixer was introduced that works by its expansion and contraction and yields steady periodical reciprocating flow. Implementation of micromixer reduced the mixing time of liquids from about 3 hours to 23 s. This study revealed that centrifugal microfluidic platforms developed in this study successfully detected dengue virus and shows better sensitivity than traditional ELISA [62]. Thiha et al. miniaturized sandwich ELISA method on lab-on-compact disk (LOCD) for the detection of dengue IgG antibody. LOCD was established by integrating microfluidic platform on a compact disk-like structure for performing the entire lab-based procedures, while the centrifugal force of spinning

disk was employed for transporting the fluid from one chamber to another. The main reason behind choosing this LOCD platform was the low cost, rapid detection, fully automation, and multiplex detection of target analytes. In addition, this platform provides high surface area to volume ratio and micromixing facility which enhance the biosensing of the assay in terms of sensitivity and specificity. After the successful development of the assay, it was evaluated by detecting dengue IgG antibody from the several hospitalized patients. The study claimed that LOCD successfully detected dengue IgG antibodies with 95% sensitivity and 100% specificity [63]. Using the microfluidic dielectrophoresis platform, Iswardy et al. developed a bead-based immunofluorescence assay for the detection of dengue virus. During the assay development, mouse anti-*Flavivirus*-capture antibodies were modified with beads, and DENV was modified with fluorescence label. The principle of this assay was based on employing the DEP to capture modified beads in the microfluidic chip which will later interact with modified DENV to form immune complex on these beads. Fluorescence microscopy was used to detect fluorescent signals, and later these signals were quantified by Image J freeware. It was found that incorporation of microfluidic platform speeds up the immuno-reactions and target analyte was detected in a short period of 5 minutes. Interestingly, this assay used ~15 μL of dengue sample to test the dengue virus presence. The study showed that the developed assay was able to detect DENV with a LOD of 10^4 PFU/mL [64].

2.1.4. Novel paper-based diagnostic devices

Paper offers several unique advantages than conventional device materials such as power-free liquid transport through capillary force and evaporation, high surface area to volume ratio, and storing reagent in active form within the fiber network [65, 66]. Lo et al. combined RT-LAMP with paper-based diagnostic devices for the detection of dengue virus. First, cDNA was amplified using RT-LAMP at 63°C. Later, the amplified products were mixed with detection probes and then moved in paper-based test zone constructed on paper-based diagnostic device. Afterwards, the fluorescent signals were examined and analyzed by image recoding system and Image J. The developed assay demonstrated high sensitivity in paper-based diagnostic device with a LOD of 31.75 μg/mL of amplified products [67]. Zhang et al. worked on to improve the flow of salivary fluid in paper-based immunoassay. This study believes that paper-based immunoassay is more often compromised due to the formation of aggregates between conjugates and specimen and, thus, inhibits the labeled target molecule to reach at test line. To resolve this issue, this study developed a stacking flow immunoassay to detect dengue-specific IgG antibody in salivary fluid. The stacking flow architecture was aimed to bypass the sample pretreatment step which is often required for testing the salivary fluid. To achieve this goal, two different paths were designed for guiding the sample and reagents separately in the test strip. According to study, application of this tactic prevented the interference of salivary substances with particle-based sensing system, and these substances were omitted before making any contact with the detection reagents, therefore, resulting in low background. Moreover, study showed equipping the strip with flow regulator enables the uniform flow in the strip which produces even test line. It was found the developed immunoassay successfully detected the dengue IgG antibodies which are important biomarker for the secondary dengue infection [68]. As we know that low sensitivity is a great disadvantage of

lateral flow assay. In one study, Kumar et al. worked on to improve the sensitivity of a paper-based assay for the detection of NS1 antigen. They exploited tapered nitrocellulose membrane and gold decorated graphene oxide sheets as the detection labels for enhancing the sensitivity of the assay. The study showed that lateral flow could detect dengue NS1 antigen with a LOD of 4.9 ng/mL using this novel format [69]. Theillet et al. developed laser-cut paper-based diagnostic device for detecting the dengue NS1 antigens and IgM antibodies. Laser cutting is a substitute pattern for producing paper analytical devices (PAD). The important aspect about this method is that any layer material could be patterned, irrespective of the fragility or thickness size of the layer. Moreover, the reproducibility pattern using laser cutting is outstanding. Laser cutting also provides the facility of modulating fluidics inside the paper channel. The purpose of using porous and hydrophilic glass-fiber paper was the motion of fluid specimen via passive capillarity. During the assay development, PADs were modified with anti-NS1-capture antibodies and EDIII antigen to capture the NS1 antigen and dengue-specific IgM antibodies, respectively. Later the plasma samples obtained from patients having acute dengue were tested on PAD as well as on LFA, for comparing the sensitivity. Detection with this type of paper-based device is found to be successful for both NS1 antigen and IgM antibody. LOD for the NS1 antigen was 25 ng/mL and it was comparable with commercial LFA [70].

3. Conclusion

In this chapter, we discussed the advanced diagnostic methods for the diagnosis of dengue. In terms of analytical sensitivity and rapidity, these novel methods showed remarkable achievements. However, most of these studies ignored the specificity criteria of diagnostic test. Specificity is a very important aspect of diagnostic test which discriminates true negative from false positive and true-positive from false-negative detection of an infectious disease. So, this aspect should be also investigated along with the sensitivity of the diagnostic test. In addition, diagnostic sensitivity and specificity should be also investigated using well-referenced samples. Moreover, all the four serotypes should be tested to see whether this method is equally effective for all serotypes. Testing of these parameters of diagnostic test will give much broad picture to analyze its potential for evolving as a point-of-care test.

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Conflict of interest

The authors declare no conflict of interest.

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