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Laboratory Diagnosis and Potential Application of Nucleic Acid Biosensor Approach for Early Detection of Dengue Virus Infections

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Dengue fever is caused by the dengue virus, the genus of Flaviviridae virus family. Until now, there is no specific medication to kill the dengue virus and patients just solely depend on the treatment of the dengue infection symptoms. Thus, a highly sensitive and rapid diagnostic tool for early diagnosis of dengue virus is very desirable, especially in resource limited-condition. We briefly review pro and cons of existing diagnostic methods for the detection of dengue virus (virus isolation, PCR, NS1Ag, Serology). We also highlight the recent advances of the biosensor technology in the dengue diagnostic dengue as a promising point-of-care diagnostic in the future. The DNA based biosensor technology combined miniaturized sample preparation offers a good opportunity for the commercialized point of care testing for dengue diagnosis in the future.

Keyword: Dengue infections, Point of care, Rapid diagnostic test, Laboratory diagnosis, biosensor technology.

About 50% of world's population or 2.5 billion people may have the potential risk of dengue infection with the number of 50-100 million cases of dengue reported annually including approximately 500,000 of dengue hemorrhagic fever cases and 22,000-24,000 deaths[1, 2]. For the past 50 years, there are four different strains of dengue virus referred as dengue serotypes type 1, 2, 3 and 4, which is transmitted to the human through the bite of female mosquitoes, Aedes *aegypti*. In October 2013, a new dengue serotype fifth has been announced as the result of blood's patients screening from the hospital of Sarawak state, Malaysia[3, 4]. Dengue virus infection caused by any of these serotypes can be asymptomatic infection (mild) or displayed in the range of severity disease from undifferentiated febrile fever and classical dengue fever to the more severe fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DHS)[5, 6]. Until now, there are no fully established specific vaccine or antiviral for dengue infection[7, 8] and most of the treatments only rely on the symptoms and a good maintenance fluid replacement to patient's body[9]. Therefore, the

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effective ways to prevent the dengue infection is to control the spread of mosquito vector by reducing and destroying the mosquito vector population[10].

Diagnostic methods of dengue virus

An early dengue diagnosis becomes important for the confirmation of dengue virus infection, clinical treatment, dengue outbreaks surveillance and monitoring efficiency of dengue vaccine trials. The clinical diagnosis of dengue infection commonly depends on reported syndromes and physical examination, which are not effective. This is because the dengue symptoms are not specific, and it is difficult to differentiate it with other infections such as malaria, West Nile, leptospirosis, measles, influenza, yellow fever and St Louis encephalitis at the early stage of infection[11, 12]. For this reason, several types of laboratory diagnostic tests have been done to confirm the dengue virus infection such as virus isolation, genome detection, virus antigen detection and specific antibody (serology) from patient's serum[13-15]. In general, dengue diagnosis is classified into two approaches of dengue virus detection; early detection and indirect detection

Virus isolation

For the past century, virus isolation technique remains as a "gold standard" for diagnosing dengue virus infection[16, 17]. The dengue virus may be recovered from infected samples such as whole blood, serum, plasma and tissues (acute samples, 4-5 days) before inoculating it into either 1 to 3-day baby mice, mosquito or cell cultures (mosquito's cell lines, C6/36 or mammalian cell lines, LLC-MK2, BHK-21 cells). This is followed by dengue virus confirmation tests using immunofluorescence (IF) technique with a specific monoclonal antibody or FTIC-conjugated anti-flavivirus antibody under UV microscope[18, 19]. Mosquito inoculation has been reported as the most sensitive for dengue virus isolation technique in comparison with cell cultures and baby mice inoculation[20, 21]. However, the disadvantages of this isolation technique are; it required high technical skill for dengue inoculation, include tedious steps and need high maintenance cost.

Antigen detection

In recent years, the detection of nonstructural protein 1 (NS1) has gained attention in the development of dengue diagnostic tests[17, 22]. NS1, a highly-conserved glycoprotein (445KDa) which was produced during dengue virus replication and circulated in bloodstream or serum at high concentration during acute infection[23-25]. According to Libraty *et al*. [26], high level of NS1 in plasma has been found in DHF patients in comparison with DF patients. However, the level of circulating NS1 in serum, plasma or whole blood will decrease with antibodies production of IgM and IgG after 5 days' infection making NS1 detection applicable at early acute dengue infection. Some of commercial diagnostic tests based on NS1 detection employing immune-chromatographic (ICT) and Enzyme-linked immunoassay ELISA methods have been developed for dengue diagnosis (Table 1). The disadvantages of this tests are unable to differentiate among dengue serotypes due to NS1 glycoprotein that is highly conserved for all dengue serotypes[27]. As anti-NS1 antibodies that are present more frequently in secondary infection, the sensitivity of NS1 assays for secondary infection is lower than in primary infection as reported by previous literature[24, 28-31]. As shown in Table 1, most of the commercial NS1 tests exhibit a poor sensitivity of NS1 detection ranging from 37-68%.

Genomic detection

Nucleic acid detection using reverse-transcriptase polymerase chain reaction (RT-PCR) protocol by amplifying the specific region of interest gene has become a powerful tool for early diagnosis of dengue virus due to the ability in providing a better sensitivity, more specificity and rapid result (less than 24 hours)[40-42]. Furthermore, the RT-PCR technique shows higher sensitivity and is cheaper than dengue detection gold standard, virus isolation technique [43-45]. By using specific primers in polymerase chain reaction, the dengue virus serotypes can be determined and differentiated allowing this technique to be highly suitable for the detection of new dengue serotypes in endemic areas.

RT-PCR technique involves three steps; RNA extraction, cDNA gene amplification, detection and characterization of amplified cDNA gene. The RT-PCR procedure starts from the extraction of RNA dengue virus from human samples (serum, plasma, whole blood, urine or saliva) followed by DNA transcription process where RNA extract is converted into complementary DNA (cDNA) using reverse transcription enzyme before cDNA amplification

process is performed[46]. According to Chen *et al*[47], the analysis of amplified cDNA gene by PCR-based technique can be performed in three-technique: a) agarose gel electrophoresis b) southern blot methods c) ELISA technique. Several modified PCR-based technique has been established for the diagnosis of dengue infection such as real-time RT-PCR[48-50], nucleic acid sequence-based amplification (NASBA)[51] and most recently, the loop-mediated isothermal amplification (LAMP)[52].

Serology tests

After 5 days of illness, serological methods are the best choice for dengue diagnosis, which relies on dengue antibodies detection of IgM and IgG. It is important to note that these antibodies, IgM and IgG are not produced at early acute of dengue infection and can be found in serum, plasma, saliva and blood. Different responses of antibodies level are observed and measured where the antibodies of IgM and IgG rise rapidly in primary and secondary infection. Several serological methods have been established for dengue virus detection including the hemagglutination inhibition test (HI), Neutralization test (NT), MAC ELISA and IgG ELISA. Among these tests, IgM/IgG assays using ELISA and Immune-chromatographic (ICT) technique are commonly used for dengue diagnosis due to simple operation without the use of the sophisticated machine, rapid results, inexpensive and reasonable sensitivity[53, 54]. However, high cross-reactivity with other flaviviruses and inability to determine the dengue serotypes become the major limitations in this dengue diagnosis as it can lead to false positive/negative results such as leptospirosis and rheumatoid arthritis[27, 55]. A few commercials of ELISA IgM and IgG assays are available for dengue diagnostic (Table 2) where IgM commercial assays exhibit the range of sensitivity of 20.5% to 77.8% and specificity of 86% to 100%. For commercial IgG assays, the sensitivity is in the range of 66% to 94.4% while specificity in the range of 86% to 100%. Summary of the pro and cons for all dengue diagnostic tests are listed in Table 3.

Biosensor as new approach in clinical diagnostic

The biosensor application in clinical diagnostic may overcome the limitation of the existing dengue diagnostic-based laboratory method, which allows the continuous monitoring, rapid response, cost-effective method, high sensitivity and specificity and easy operation with minimum sample preparation. The biosensor can be defined as an analytical device incorporated with sensing material and molecular recognition elements such as an enzyme, protein antibodies, nucleic acid, hormone, chemical compounds integrated within transducers[62, 63]. The basic principle of this biosensor detection relies on the interaction between molecule recognition in sensor devices with its specific target and the change of this biochemical is translated into quantifiable signal responses via the transducer, whether in the form of electrochemical[64, 65], electrical[66, 67], optical[68, 69] or piezoelectric[70].

Nucleic acid-based biosensor in dengue virus detection

With the demand for rapid, simple and point of care (POC) tests in clinical diagnostic, there have been growing interests in exploring biosensor application for dengue virus detection[71-79]. Most of them applied nucleic acid aptamers (genosensor) as the molecule recognition element in their fabricated DNA biosensor for dengue virus detection by the hybridization process [80]. Nucleic acid aptamers are a short sequence of synthetic oligonucleotide (single-stranded DNA or RNA) approximately 25-100 bases with the specific sequence that can recognize and bind with high affinity to its nucleic acid target[80-82]. In general, the nucleic acid hybridization detection via biosensor approach is based on a duplex of nucleic acid formation (DNA-DNA, DNA-RNA) as the results of two single-stranded nucleic acids are combined between nucleic acid aptamers with specific sequence (known as DNA probe) with its specific complementary sequences (known as DNA target)[83, 84]. Using a suitable hybridization indicator, this duplex DNA hybridization can be detected and converted into a measurable signal including optical, electrochemical, electrical and piezoelectric.

In our previous work , we successfully constructed a simple fabrication of DNA dengue electrochemical biosensor where DNA probes are immobilized (23 bases) on the surface of indium tin oxide (ITO) and screen-printed gold electrode (SPGE), respectively before allowing hybridization with its complementary DNA dengue sequences[80, 81, 84]. The DNA-DNA hybridization detection was

monitored using the electrochemical method based on methylene blue oxidation. The RNA dengue virus detection based on DNA/RNA hybridization using optical biosensor approach has been shown in the work of Kwakye *et al*[85]. In their studies, two different DNA probes were used; the DNA probes functionalized liposome and reporter probe tagged with magnetic beads were hybridized with amplified RNA dengue target forming a hybridized DNA/RNA complex-liposome that can be visualized under fluorescent microscopy. The interesting fact about their work is the level of RNA virus concentration can be estimated and it also managed to achieve 10 times lower of the limit of detection (LOD) of RNA dengue virus than of conventional laboratory methods. Nascimento *et al*[86] had utilized the gold nanoparticles-polyaniline hybrid composite layer as DNA matrix in their fabricated DNA sensor to improve probe loading which in turn increased the DNA target loading and thus, resulted in the high sensitivity and able to discriminate the dengue serotypes 1,2 and 3 at the picomolar concentration of DNA dengue virus detection. The incorporation of RT-PCR methods in DNA biosensor for rapid detection of dengue virus has been reported by Rai *et al*[75]. In their work, a nanop orous alumina membrane biosensor was constructed where the DNA probes are immobilized inside the alumina channel and upon hybridization with amplified RT-PCR products, the reduction of the electrochemical signal occur indicating successful detection of dengue virus. In this strategy, the RT-PCR technique is used to amplify the level of concentration of genomic nucleic acid

Table 1. The commercial of dengue diagnostic tests based on the NS1 detection

Commercial NS1 diagnostic test	Sensitivity	Specificity	References
ELISA technique			
Platelia Dengue NS Ag-ELISA	68%	96%	[32]
Platelia Dengue NS Ag-ELISA	37%	100%	[33]
Platelia Dengue NS Ag-ELISA	45%	92%	[24]
PanBio Dengue Early ELISA kit	65%	98%	[23]
PanBio Dengue Early ELISA kit	45%	92%	[24]
PanBio Dengue Early ELISA kit	69%	96%	[34]
PanBio Dengue Early ELISA kit	66%	100%	[35]
Dengue NS1 Elisa	65%	98%	[36]
Immune-chromatographic (ICT) technique			
PanBio NS1 antigen strip	46%	98%	[37]
Dengue NS1 Ag Strip kit	49%	99%	[38]
Dengue NS1 Ag Strip kit	51%	97%	[39]

Table 2. The commercial of IgM and IgG assays for dengue diagnosis

Commercial of IgM/IgG assays	Sensitivity	Specificity	References
IgM assays			
SD Bioline IgM (ICT)	61%	90%	[56]
Zephyr IgM (ICT)	20.5%	86%	[56]
SD Duo IgM (ICT)	53.5%	100%	[36]
Omega capture ELISA	62.3%	97.8%	[56]
Venture IgM ELISA	68.7%	100%	[57]
PanBio IgM ELISA	77.8%	90.6%	[41]
IgG assays			
PanBio IgG capture ELISA	81.2%	63.5%	[58]
Panbio Dengue Duo cassette (ICT)	66.4%	94.4%	[59]
Panbio Dengue Duo (ICT)	87.5%	66.6%	[60]
SD Bioline IgG	90.06%	92.48%	[61]

dengue virus before it can be detected by their fabricated DNA biosensor. This combination of PCR-DNA biosensor can replace the conventional PCR products analysis, which relies on agarose gel electrophoresis and southern/northern blotting. The use of agarose gel electrophoresis analysis exhibit low sensitivity and does not provide specific sequence information of amplified nucleic acid[87]. Besides that, this conventional PCR product analysis is usually time-consuming, consist of tedious steps requires specialized equipment and hazardous elements such as ethidium bromide and ultraviolet[88, 89]. Another study of DNA biosensor coupling with RNA target amplification based PCR technique was reported by Baeumner *et al*[79]. Based on their work, the RNA extract was first amplified using isothermal nucleic acid sequence-based amplification (NASBA) for DNA/RNA hybridization using liposome amplification signal. The first reporter probe tagged with liposome was mixed and hybridized with the amplified RNA before being introduced to the surface of a polyethersulfone membrane trip containing immobilized DNA probe. With this DNA/RNA hybridization detection, the amount of RNA dengue virus was correlated with the amount of liposome that can be measured using a portable reflectometer. They have found that their fabricated DNA biosensor could detect the RNA dengue virus concentration at low concentration of 10 PFU /mL.

Recently, some studies have employed dengue monoclonal antibodies based biosensor (also known as immunosensors) which are based on antibodies-antigen interaction for the detection of dengue biomarker proteins such as NS1[90, 91] and IgM[92]. However, the application of DNA biosensor is more advantageous and can be an alternative compared to immunosensors based on few reasons. In contrast to antibodies, nucleic acid aptamers (DNA probe) are more easily synthesized in a large-scale production which can offer high sensitivity and specificity, high stability (pH and temperature), long shelf life, and can be regenerated after the heat-denatured process of hybridized DNA[82, 89, 93]. Besides that, the production of nucleic acid aptamers is a cost-effective and less time-consuming as compared to antibodies which rely on expensive animal hosts to produce antibody up to 6 months[94, 95]. As aptamers are easily modified and conjugated with labelled/reporter molecules compared to antibodies, making DNA biosensor is more versatile than immunosensors.

Table 3. Summary of pro and cons for current diagnostic tests for dengue infection

Diagnostic tests	Advantages	Disadvantages
Virus isolation	Specific Reliable results	Time consuming (6-10 days) Lower sensitivity Expensive Laborious steps Require appropriate facilities and expertise skills Require acute sample (0-5 days)
RT-PCR technique	High sensitivity High specificity Results less than 24 hours Reliable results	Expensive Require specialized instrumentation Require careful handling to avoid cross-contamination during the RT-PCR procedure Involve hazardous chemicals Require acute sample (0-5 days)
NS1 detection	Inexpensive Rapid results (In a few hours) Easy to operate	Lower sensitivity High cross-reactivity with another antigen/antibody False positive/negative results) Require acute sample (0-5 days)
Serology test (IgG and IgM)	Inexpensive Rapid results (In a few hours) Easy to operate	Lower sensitivity High cross-reactivity with other flaviviruses and antigen/antibody False positive/negative results Require febrile sample (5 days above)

Furthermore, the smaller size of nucleic acids than antibodies allow high-density monolayer of aptamer recognition which gives advantages in the miniaturization of the biosensor[96, 97].

Conclusions and Outlook

In this review paper, we discuss the pro and cons of existing conventional dengue diagnostic methods. Most conventional diagnostic methods are still based on laboratory methods, time-consuming, require sophisticated instrumentation and trained person to diagnosis dengue infections which are not suitable in resource-limited condition. Although, dengue rapid test (RDT) based immunochromatography (NS1 antigen and IgM) are available in the market that can eliminate the use of laboratory equipment, but it is more suitable for initial screening to identify whether a person is infected or not before need a further confirmatory test. This is attributed that commercial RDTs have a high risk of cross-reactivity with other flaviviruses leading to false-positive results. Moreover, the lack of sensitivity is often a major challenge in dengue diagnostic based RDT approach. DNA-based biosensor technology has gained an attention by many researchers for its potential to replace the conventional dengue diagnostic method due to its rapid diagnostic as early as one day, cost-effective, highly sensitive and specific, easily miniaturized and can be operated in resource-limited condition. Interestingly, monitoring the level of nucleic acid of dengue virus concentration in patients can provide a valuable information about the progression of dengue virus infection, thus effective treatment could be achieved. To date, there is no DNA-based biosensor for dengue virus cases detection have been commercialized yet. One of the possible reason is the complexity of clinical samples that require bulky instrumentation for sample preparation assays before being detectable by biosensor that could limit the application in point-of-care testing. To date, much effort to put towards the miniaturized sample preparation assays that hold promise to be integrated with DNA biosensor in one single system such as microfluidic or lab-on-a-chip, giving opportunities for commercialized point-of-care testing for dengue diagnosis. We believe this DNA-based biosensor technology combined miniaturized sample preparation seems compatible with the internet of things (IoT) application that could revolutionize healthcare especially in dengue diagnostic.

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