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Future Perspective of Nucleic Acid-based Detection of Dengue Virus and its Serotypes

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Abstract

Dengue is an arthropod-borne viral disease of tropical and subtropical areas. A research group has estimated that there are 390 million dengue infections per year, 96 million in whom symptoms manifest. The symptoms of a primary infection with dengue are usually mild and resolve with time, but secondary infection with dengue virus of a different serotype may lead to more severe symptoms such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). No vaccine or antiviral drug is approved for the prevention or treatment of dengue diseases. Therefore, it is important to diagnose the infection and differentiate the serotypes as early as possible. Currently, immunochromatographic assays are used for the diagnosis of clinical specimen from dengue infected patients in hospital, since they can detect the target antigen within 15min without any facilities. On the other hand, nucleic acid-based diagnostic assays such as reverse transcription-polymerase chain reaction (RT-PCR), reverse transcriptase loop-mediated isothermal amplification (RT-LAMP), and nucleic acid-chromatography also are useful for assessing dengue virus infection and virus serotypes in the early stages of infection in a sequence-specific manner. Diagnostic methods that possess advantages of both immunochromatographic assays and nucleic acid-based diagnostic assays should enable us to diagnose dengue virus infection in a rapid and serotypes-specific manner. This paper discusses the advantages and the future prospective of currently developed nucleic acid-based lateral flow assays and their applications in point-of-care (POC) testing kits.

Keywords: Dengue virus; Serotypes; Peptide nucleic acid (PNA); TaqMan probe; Loop-mediated isothermal amplification (LAMP); Lateral flow chromatographic assays; Nucleotide-based assay; Systematic evolution of ligands by exponential enrichment (SELEX); Nucleic acid sequence based amplification (NASBA)

Opinion

Dengue virus

Dengue viruses (family *Flaviridae*, genus Flavivirus) have four distinct antigenic types (serotypes 1 to 4). The dengue virus RNA genome is a single-stranded positive-sense genome of approximately 10,700 bases, and is packaged in capsid proteins and covered by a lipid envelope containing membrane proteins [1]. These viruses are maintained mainly via human-*Aedes aegypti* and human-*Aedes albopictus* mosquito transmission cycle, primarily in tropical urban areas, and are currently endemic in most tropical areas of the world [2]. Following introduction into human skin (via mosquito bite), dengue viruses infect immature dendritic cells. These infected cells mature and migrate to local or regional lymph nodes, initiating cellular and humoral immune responses [3]. Subsequently, infected cells and activated endothelial cells produce tumour necrosis factor α and nitric oxide, factors that increase vascular permeability and induce plasma leakage in patients with dengue haemorrhagic fever 3-7 days after onset [4]. Therefore, early-stage diagnosis and treatment are crucial to the prevention of fatal cases.

Detection of dengue virus by one-step SYBR Green I-based reverse transcription-PCR (RT-PCR) assay

For the detection and typing of dengue virus in patient serum samples by this PCR-based method, the initial step is the extraction of the viral RNA from acute serum using a commercially available viral RNA purification kit. The viral RNA then is reverse transcribed into complementary DNA (cDNA) by employing serotype-specific primers and M-MLV reverse transcriptase. Subsequently, cDNA is amplified by real-time PCR, using a SYBR Green PCR kit with primers specific to each serotype [5,6].

SYBR Green has high binding affinity for the minor groove of dsDNA. The compound exhibits fluorescence when bound to dsDNA, making the dye useful for the detection of elongated dsDNA products during real-time PCR. Therefore, this technique is used to estimate the initial level of viral RNA in the specimen. The advantage of this RT-PCR-based method is high sensitivity, providing a detection limit of 10 to 100 copies [7]. However, given that SYBR Green dye will detect *any* double-stranded DNA, additional testing (such as melting temperature analysis or inspection of specific size of amplicon by agarose gel electrophoresis) is required to confirm the generation of a specific amplicon.

Detection of dengue virus by TaqMan real-time one-step RT-PCR assay

The TaqMan real-time one-step RT-PCR assay was developed to enhance the sequence specificity of the one-step SYBR Green I-based reverse transcription-PCR assay. The TaqMan probes consist of an oligonucleotide probe with a reporter dye attached to the 5' end and a quencher dye attached to the 3' end. During PCR, the probe anneals, specifically between the forward and reverse primers, to an internal region of the PCR product. The polymerase then carries out

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the extension of the primer and replicates the template to which the TaqMan is bound. The 5' exonuclease activity of the polymerase cleaves the probe, resulting in a liberation of reporter dye from quencher which increases fluorescence emission. The TaqMan probe has been shown to improve the specificity of diagnosis up to 100% relative to a virus isolation assay [8].

Detection of dengue virus by loop-mediated isothermal amplification (RT-LAMP) assay

The loop-mediated isothermal amplification assay has been used as a very sensitive, easy to use, and less time-consuming diagnostic method. LAMP can amplify up to 10^9 copies in less than 1 h under isothermal conditions (65°C) using simple incubators (e.g., water baths or heating blocks), making this approach suitable for field work. This system employs 4-6 primers that recognize target DNA, thereby helping to eliminate non-specific binding and efficiently discriminate dengue virus of serotypes 1-4 [9]. The genome amplification step is monitored by the visible colour change of HNB (hydroxylnaphthol blue) from violet to sky blue as the LAMP reaction proceeds [10,11]. This method can detect approximately 10-copy templates of dengue virus [12]. However, subtyping of each sample requires the use of either a set of four tubes, each of which has the primer mixture for a particular serotype [9], or of one tube that has a single reaction mixture incorporating all primer sets [11].

Detection of dengue virus by lateral flow assays

Serological tests are commonly used for the detection of antibodies in the serum and other body fluids in order to diagnose active or previous dengue virus infection. IgM, IgG, and NS1 antigen can be used in serological detection. The IgM antibody is detectable within 3-5 days of onset and may persist for months, making this antibody a good marker of recent infection [13]. The IgG antibody response develops within a few days after the onset of IgM antibody, and is detectable for 6 weeks [14]. Patterns of antibody response differ between primary and secondary infection, with primary dengue evoking stronger and more specific IgM response than secondary infection, which induces a stronger and more rapid IgG response. Prior vaccination against other flavivirus or prior infection with non-dengue flavivirus potentially influences antibody responses as measured in some assays [15]. The detection (by monoclonal antibody) of secreted NS1 protein in viremia is also a new approach to the diagnosis of acute dengue infection [16]. Commercial dengue rapid diagnostic tests, which detect dengue virus NS1 antigen (Figure 1a) and IgM/IgG antibodies, have been used for early dengue diagnosis. However, to date this method does not permit differentiation of serotypes and may provide false-positive results due to potential cross-reactivity with other flaviviruses [17].

DNA aptamers are *in vitro* selected nucleic acid molecules that can bind to a broad range of targets with high affinity and specificity. The selection system used to identify aptamers is called systematic evolution of ligands by exponential enrichment (SELEX). SELEX permits the identification of high-affinity binders from large pools of randomized nucleic acids followed by enrichment through subsequent rounds of selection [18]. DNA aptamer sequences that recognize the E proteins of arboviruses (a class that includes dengue) have been obtained by an ELISA affinity-capture assay; some of these sequences then were used to detect arboviruses via an aptamer-based lateral flow chromatographic assay (Figure 1b) [19]. However, given that E-like proteins are shared among various flaviviruses, E protein cross-reactivity can affect the sensitivity of this type of assay.

Serotype-specific DNA probes also have been used to detect dengue virus serotypes [20]. In this system, a single-stranded DNA capture probe is immobilized through biotin-avidin interaction on the surface of a polyethersulfone membrane strip and used to capture dengue RNA that has been amplified by an isothermal nucleic acid sequence-based amplification (NASBA) reaction. The surface-captured RNA then is labelled with another DNA reporter probe that is coupled to dye-entrapping liposomes (Figure 1c) [21].

In other work, peptide nucleic acid (PNA)-based lateral flow assays have been used to discriminate viral subtypes [22]. Since PNAs possess high binding affinity and sequence specificity for RNA than for DNA [23], PNA-based lateral flow assays can potentially provide higher sensitivity than DNA-based lateral flow assays. In the case of influenza virus subtyping, biotinylated-PNA is mixed with the virus lysate to obtain a biotinylated-PNA/viral RNA-nucleoprotein complex. After the biotinylated-PNA/RNA-nucleoprotein complex is loaded on a lateral flow column and captured on the anti-biotin antibody-immobilized line, the complex is visualized by mouse anti-nucleoprotein monoclonal

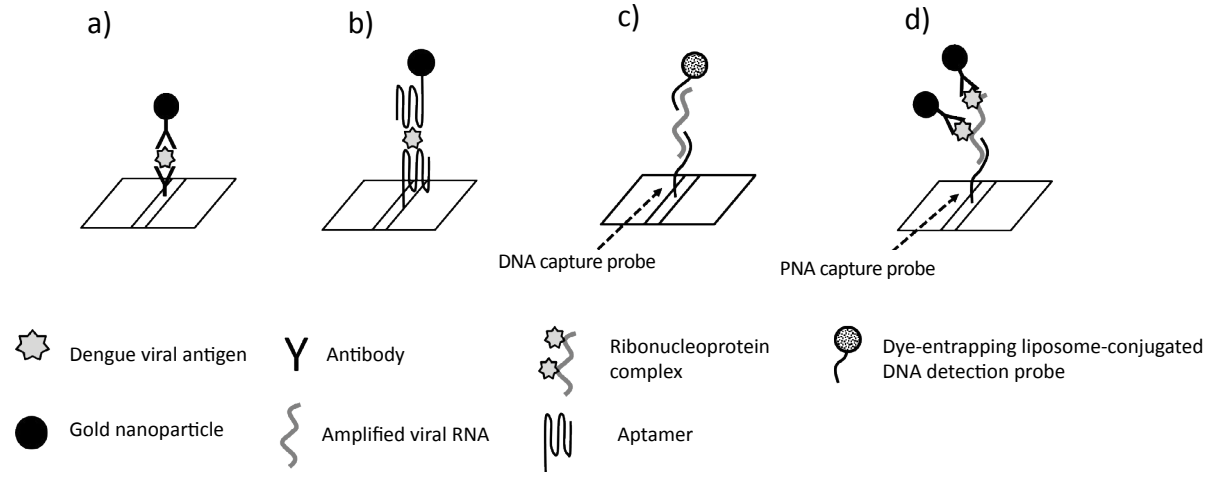

Figure 1: Detection of dengue virus by different types of lateral flow chromatographic assays. Antibody-based immunochromatographic assays, which detects viral antigen with a) a set of antibodies or b) a set of DNA aptamer. Nucleic acid-based affinity capture assays, which detect c) viral RNA with a dye entrapping liposome-conjugated nucleic acid probe or d) viral ribonucleoprotein complex with gold-conjugated antibody probes.

category	Diagnostic method	Time to results	samples	Cross-reactivity with other flaviviruses	Serotype specificity	facilities
Detection of viral nucleic acid	RT-PCR	4 h	blood or urine	Rarely	High	Thermal cycler
	RT-LAMP	2 h	blood or urine	No	High	Isothermal heater
Detection of viral antigen	Immunochromatography for NS1 antigen (Fig.1a)	15 min	blood	React with other flaviviruses	Mid	Not required
	DNA aptamer probe for E-protein (Fig. 1b)	15 min[2]	viral protein[2]	Not tested	-	Not required
Detection of viral RNA or ribonucleoprotein complex	DNA-based probe for viral RNA (Fig.1c)	30 min[3]	viral RNA[3]	Not tested	-	Not required
	PNA-based probe for virus ribonucleoprotein complex (Fig. 1d)	15 min	virus lysate[4]	Not tested	-	Not required

[1] High: specificity is higher than 95%, Mid: specificity is between 60-94%.
[2]Data was obtained using recombinant dengue virus protein. Not yet tested for dengue virus specimen.
[3]Data was obtained using dengue virus RNA amplified by NASBA. Not yet tested for dengue virus specimen.
[4]Data was obtained using influenza virus test case. Not yet tested for dengue virus specimen.

Table 1: Diagnostic test for viral RNA and protein detection.

antibody and anti-mouse IgG secondary antibody conjugated with gold nanoparticles (Figure 1d) [22]. Although the sensitivity of this method (10^4 pfu/ml) is lower than that of RT-PCR or RT-LAMP (10 copies each), PNA-based lateral flow can be used for the detection of viral genome RNA in clinical samples without prior purification and amplification.

Among these nucleic acid-based diagnostic assays, RT-PCR and RT-LAMP provide the most sensitive and specific results. However, these assays are most suitable for laboratory-based diagnosis, given the special conditions required for the performing techniques such as viral RNA purification, reverse transcription, and genome amplification with specific instruments. In contrast, nucleic acid-based lateral flow assays can be employed for bedside diagnosis of dengue virus infection, since these assays are easy to use, require small reagent volumes, do not consume energy, and permit the detection of target viral genomes in a sequence-specific manner. Further optimization of nucleic acid-based lateral flow assays is expected to provide clinicians with a new type of POC testing kit that can detect not only dengue virus infection, but also the serotype of the infecting virus.

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