Review

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Overview of dengue diagnostic limitations and potential strategies for improvement

https://doi.org/10.1515/dx-2024-0173
Received October 23, 2024; accepted December 26, 2024;
published online January 29, 2025

Abstract

Introduction: Dengue is a viral infection caused by any one of the four related dengue virus (DENV) serotypes, 1–4. DENV is a single-stranded RNA virus belonging to the genus *Flavivirus*. Dengue can cause a range of symptoms, from mild to severe life-threatening illness. Currently, treatment for DENV is limited to supportive care, with better outcomes achieved through early diagnosis. The WHO has suggested that dengue mortality can be reduced to nearly zero by implementing appropriate clinical management strategies, such as early laboratory diagnosis. This calls for diagnostic approaches that combine high sensitivity and specificity, while also being suitable for point-of-care testing (POCT) in remote locations with minimal staff training and low testing costs.
Content: In this paper, we outline the limitations of existing confirmatory dengue diagnostic methods, such as ELISA and RT-PCR, which are time-consuming, expensive, and require skilled personnel. We also highlight alternative strategies to overcome these challenges. Additionally, the paper emphasizes the growing clinical demand for diagnosing severe dengue to reduce the risk of death, which must be addressed by next-generation dengue diagnostic approaches.
Summary: We propose the adoption of alternative strategies, such as fluorescence immunoassay (FIA) and chemiluminescence immunoassay (CLIA), which have the potential to overcome the limitations of existing dengue diagnostic methods.
Outlook: Improvements in dengue diagnosis, with a specific focus on identifying severe dengue in POCT setting, can help achieve the goal of zero deaths from dengue.

Keywords: dengue; diagnosis; RT-PCR; ELISA; CLIA; FIA

Introduction

Dengue is a mosquito-borne viral disease prevalent in tropical and subtropical regions of the world. With over 100 million cases and 20,000–25,000 deaths annually, dengue poses a significant public health challenge [1]. Global incidence of dengue has surged ten-fold over the past two decades with reported instances spreading across 129 countries. Post COVID-19 pandemic, an upsurge in dengue cases have been observed globally with occurrence of multiple outbreaks and spreading into regions previously unaffected by dengue [2]. Presently, about half of the world's population is now at risk of DENV infection, and the geographical range of dengue is expected to expand due to ongoing climate change and urbanization [3, 4].

DENV is transmitted via the female *Aedes aegypti* and *Aedes albopictus* mosquito, a primary vector for the infection [5, 6]. Dengue virus (DENV) is classified under genus flavivirus and four distinctive serotypes (DENV1, 2, 3 and 4) causing human disease are currently confirmed. All four serotypes of DENV are distinct and evolved from sylvatic (or "jungle") strains whose life cycle was between forest-dwelling mosquitos and non-human primates [7, 8]. The dengue virion particle consists of lipopolysaccharide containing spherical envelope that is 40–50 nm in diameter that encloses the positive single-strand RNA genome (10.8 kb) and has an open reading frame that encodes a single polyprotein. This polyprotein is cleaved into three structural proteins (capsid, membrane and envelope) as well as eight non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS2K, NS4B, and NS5) that assist the replication of viral genome [9–11].

Dengue is largely asymptomatic and the most common outcome being acute febrile illness (dengue fever). Severe dengue illness (dengue hemorrhagic fever) is caused by

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spontaneous haemorrhage which is attributed to the formation of antibodies against viral antigen, non-structural protein 1 (NS1), that also cross-react with platelets and non-infected endothelial cells thereby triggering disturbances in capillary permeability [12]. Untreated severe dengue fever may have a mortality rate of 10–20 %. However, with appropriate supportive care, the mortality rate can be reduced to approximately 1 % [1]. Outbreaks with the mass spread of dengue, particularly in developing and underdeveloped countries, result in considerable burden on the healthcare system and economic impact. Given the current lack of an effective vaccine or approved antiviral treatment, it is likely that dengue represents an important public health problem in coming years. In this regard, diagnostic tools for the early diagnosis of dengue remain the primary tool for initiating quick disease control measures and minimizing mortality. This is reflected in WHO's sustainable development goals for 2021–2030 targeting zero deaths from dengue virus through effective diagnosis and clinical management [13]. Achieving this goal needs realization of the gaps in existing diagnostic approaches and addressing them scientifically. To this end, this review aims to compare the existing approaches for dengue diagnosis and highlight the areas of improvement that are needed to achieve effective dengue diagnosis.

Standard approaches for dengue diagnosis

Initial diagnosis of dengue is based on clinical symptoms that guide the subsequent laboratory confirmation protocol. Existing recommendations for the laboratory confirmation of dengue include NS1 ELISA or RT-PCR up to a period of first five days of illness [14]. For the confirmation of acute dengue across different sample age (days after illness) groups, NS1 antigen-capture ELISA is the most reliable approach [15]. NS1 is a glycoprotein whose levels increase rapidly on infection with DENV. It is present in serum between 1 and 9 days of illness [16, 17]. NS1 antigen-capture ELISA has been found to be more sensitive than virus isolation, conventional RT-PCR and real time RT-PCR [15]. This is also highlighted by the findings in RT-PCR negative air travelers who later turned out to be anti-dengue IgM positive [18]. The percentage of dengue positive identification by virus isolation/RT-PCR/real-time RT-PCR are higher (≥70 % in early stages of infection (days 1–3 of illness) whereas NS1 antigen capture ELISA can identify the infection over a broader range (days 0–8 of illness). Thus, positive detection of anti-dengue IgM even with a negative finding by other test methods (such as RT-PCR, real-time RT-PCR, dengue antigen and virus isolation) could still be suffering from acute dengue infection. This consideration is important for diagnosing patients who sought late treatment [15]. Thus, NS1 antigen ELISA is crucial for the early-stage dengue diagnosis. In addition, an anti-dengue IgM test is also suggested for the confirmation of acute dengue after three days of illness. This is because the initial phases of infection, Immunoglobulin M (IgM) antibodies begin to appear by 3–5 days of illness and reach peak levels in approximately 2 weeks after onset of fever and progressively decline to undetectable levels over next 2–3 months [19].

A positive detection of anti-dengue IgM using IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) can be used as an indication of acute dengue infection with period of illness more than three days. But this practice is applicable for countries where there is no endemic transmission of DENV. Whereas, in countries where dengue is highly endemic, considering a positive detection of anti-dengue IgM based on a single serum sample as an indicative of acute dengue will lead to a false positive diagnosis. In this regard, combination of IgM detection with the detection of free dengue antigen have been used for the diagnosis of acute phase of dengue infection. Since the formation of immune complexes impedes the detection of free NS1 protein [20], NS1-antigen ELISA combined with anti-dengue IgM ELISA currently caters for the standard POCT of dengue [21]. Based on these considerations from literature [15, 22], an algorithm for the laboratory confirmation of dengue is provided in Figure 1.

Apart from these diagnostic approaches, haemagglutination-inhibition (HI) test has been used for many years due to its high sensitivity and relatively easy to perform. It is of great value in Sero-epidemiological studies and differentiating primary and secondary infections based on differences in the antibody titers. However, its main limitations are poor specificity, requirement of paired sera and failure to differentiate dengue serotypes/other members of genus flavivirus [23]. Besides immunological tests, platelet and hematocrit levels are also used as an indication for acute dengue illness. This is because the drop in platelet levels below 100,000 is a common feature of dengue fever [24]. The dynamics of the different analytes used for dengue diagnosis and other clinical parameters during the course of illness are provided in Figure 2.

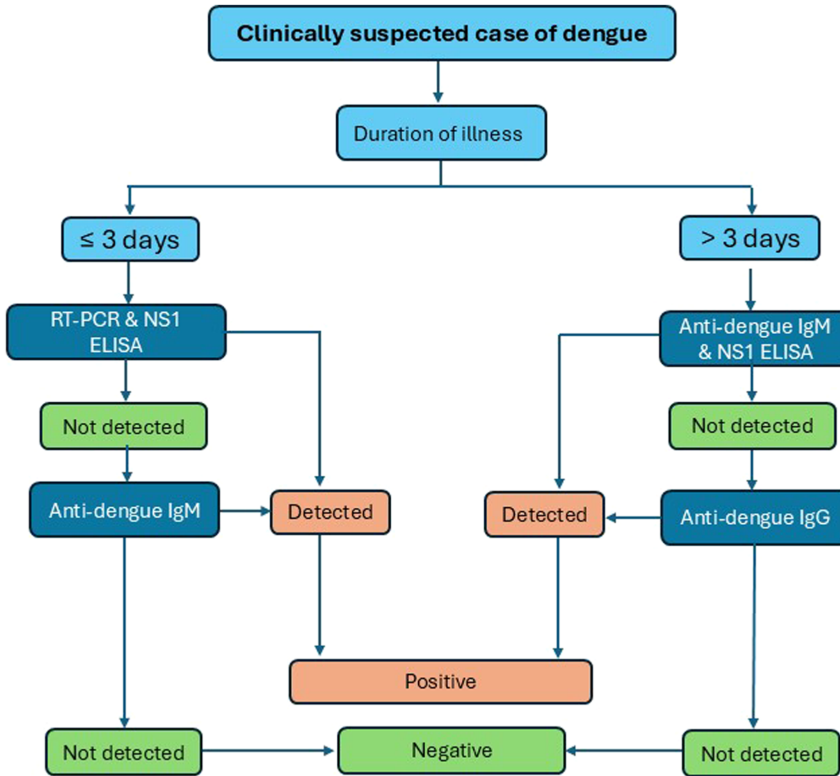

Figure 1: Algorithm for the laboratory confirmation of dengue. Dengue diagnosis involves carrying out different tests depending on the duration of illness. The algorithm is based on PAHO-WHO and CDC guidelines and considerations provided in literature [15, 22].

Course of dengue illness	Febrile	Critical	Recovery
Days	1-3	4-5	6-10
Fever	High	Low	Nil
Potential clinical issues	Dehydration	Haemorrhage, Organ impairment	Reabsorption/fluid overload
Haematological changes	Hematocrit: basal Platelet: high	Hematocrit: high Platelet: low	Hematocrit: basal Platelet: high
Serology (IgM/IgG)	IgM: Low	IgM: High	IgM: High
	IgG: Low (if no previous DENV exposure)/High (with previous DENV exposure)		IgG: High
Virology	DENV RNA: High NS1: High	DENV RNA: Low NS1: High	DENV RNA: Not detected NS1: Moderate

Figure 2: Dynamics of the dengue related parameters during its course of illness. Changes in the different parameters (associated with clinical symptoms, hematology, serology and virology) under acute dengue illness are listed according to the three stages of illness (febrile, critical and recovery).

DENV-related factors that demand advancement in dengue diagnosis

The laboratory diagnosis of DENV has largely improved over the years. However, given the fact that clinical presentation of dengue fever often overlaps with the presentation of other different flaviviruses that continue to spread globally, specific diagnosis of DENV in a "point of care" setting remains a critical challenge. Each of the four DENV serotypes have only around 65 % similarity with each other, but yet produces nearly identical syndromes in humans [25]. Though infection with a particular serotype confers lifetime immunity to that particular serotype, subsequent infection with a different serotype increases the severity of the disease due to a process known as antibody-dependent enhancement (ADE) of infection [26, 27]. In this scenario, early initiation of aggressive intravenous rehydration therapy is needed [19], and hence, information regarding the serotype is clinically useful to reduce the complications from dengue. In contrast to the disease severity associated with pre-exposure to particular DENV serotype, seronegative human subjects are at a risk of severe dengue hospitalization when they are receiving the dengue vaccine. This has led to the recommendation for pre-vaccination screening for dengue serostatus during clinical use of dengue vaccines. In this regard, WHO has highlighted the urgent need for lateral flow immunoassay or rapid diagnostic tests (RDT) to determine DENV serostatus, which is currently lacking [28, 29].

Cross reactivity of the DENV diagnosis with other members of flavivirus has also been an aspect of concern especially in regions where they cocirculate in the dengue endemic regions and exhibit related symptoms [30]. In this scenario, a poor performance in dengue detection specificity has been observed even though the detection specificity is above 90 % in dengue non-endemic regions [31]. Besides, non-specific reactivity has also been observed in sera from patients with malaria in anti-dengue IgM ELISA test warranting further improvement in dengue diagnostic tests where malaria is endemic [32]. In this regard, identification of epitopes that retain the existing high sensitivity to detect acute and convalescent phases of infection while providing acceptable specificity is needed [21]. Besides cross reactivity of the epitopes, the wild-type NS1 expression in different cell lines is also different between serotypes (with DENV1 and DENV3 having the highest and lowest wild-type NS1, respectively). In addition, it is also important to take into consideration the recently emerging NS1 mutations such as Val236➔Ala (DENV2) or Trp68➔stop codon in DENV3 which result in NS1-ELISA negative results despite being positive in RT-PCR [33]. These aspects are to be considered for standardizing development of newer dengue diagnostic approaches that can limit the risk of death due to severe dengue from ADE.

Assay related factors that demand advancement in dengue diagnosis

The plaque reduction neutralization test (PRNT) is considered a gold standard serological test to differentiate the infections from different members of the genus flavivirus [34]. PRNT involves serial dilutions of the serum that are mixed with a standard amount of virus. The amount of unbound virus in the mixture is then tested by measuring viral infectivity by counting formation of localized virus infected areas (plaques) in susceptible cells grown on semi-solid media [35]. However, PRNT is labor-intensive, requires live viruses maintained under good laboratory practice conditions, and is susceptible to conditions such as differences in cell lines used, maturation state of the virus etc. [36]. These factors limit the practical utility of PRNT [21].

RDTs have been largely used for the detection of IgG/IgM in serum. Simplicity in the use of RDT with limited skills has been its major advantage. Comparison of different commercial RDT performances with ELISA have shown sensitivity and specificities between 80 and 100 % [37]. However, RDT suffers from user-dependent variation in visual result-interpretation, lack of quantitative outcome, and not being able to discriminate DENV serotypes [38, 39]. On the other hand, ELISA is limited by its time-consuming steps such as sample-dilution which require skilled manpower to ensure that the readings lie within the linear range of the assay, which in turn is limited by the optical density (OD) limit in colorimetry [40]. This also limits its scope for automation thereby affecting its scaling-up to high-throughput operation.

Single-tube multiplex RT-PCR that determines all four serotypes have been developed. However, the reaction is five-fold less sensitive compared to the RT-PCR [41]. This is attributed to the possibility of spurious amplification products from the formation of dimers due to more numbers of primer pairs [42]. Besides, amplicon contamination could also yield false positives and demands highly experienced and skilled personnel to carry out RT-PCR. Moreover, RT-PCR based testing is also limited by its high costs for procurement and maintenance in resource-constrained distant areas where dengue is endemic. Alternative strategies for the detection of DENV RNA without thermocycling (used in RT-PCR) such as isothermal nucleic acid-sequence based

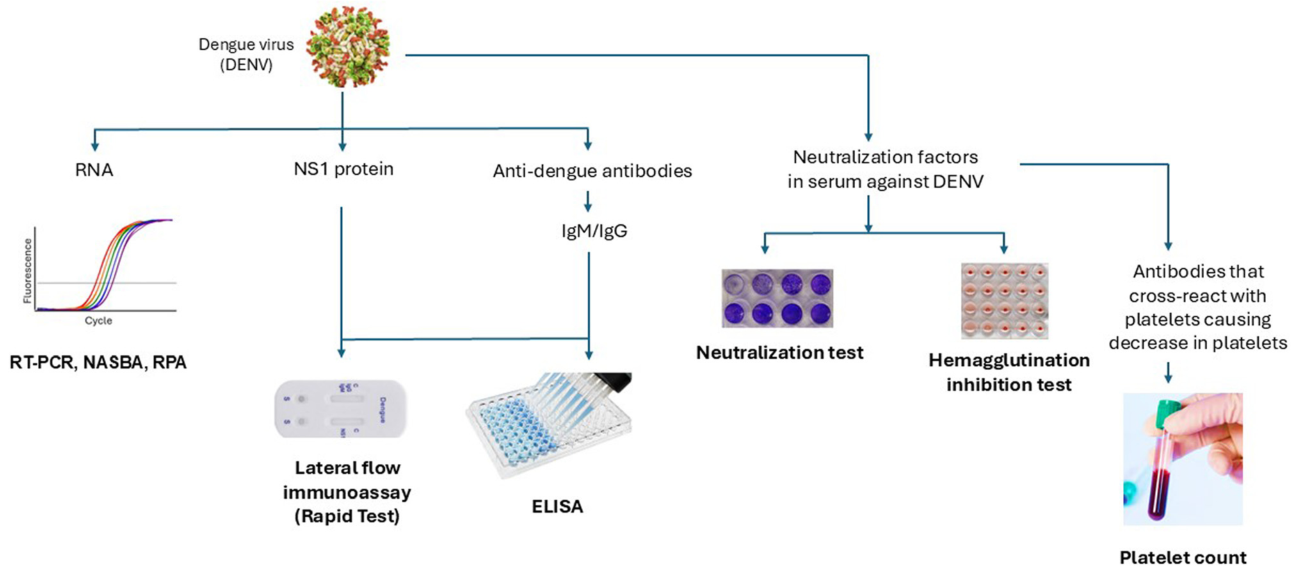

Figure 3: Overview of dengue diagnostic methods. Dengue diagnosis involves the determination of the presence of different viral components (viral RNA, NS1 and envelope proteins) and/or immune response factors (IgM/IgG/viral neutralization factors in serum). RT-PCR: reverse transcription polymerase chain reaction; NASBA: nucleic acid-sequence based amplification assay, RPA: recombinase polymerase amplification.

amplification assay (NASBA) and real-time recombinase polymerase amplification (RPA) have also been developed. They have the advantage of being able to detect DENV serotypes at a lower cost than RT-PCR. However, these strategies require the extraction of purified RNA that requires trained personnel, specialized lab facility and equipment. An overview of the different dengue diagnostic methods based on their targeting towards different dengue-specific analytes is provided in Figure 3. Collectively, though the existing tools for dengue diagnosis have the sensitivity and specificity to detect a positive dengue infection, they still lack the ability to distinguish the serotype of dengue infection or other flavivirus infections in a POCT setting. A summary of the advantages and limitations of the different analytes and detection methods used for dengue diagnosis are outlined in Table 1.

Current trends and needs in dengue diagnosis

Currently, there is no way of telling whether a patient with dengue will develop severe dengue or not. This demands to admit patients in hospital and monitor their platelet and hematocrit levels at regular intervals that puts additional burden on the health care system, especially during peak dengue seasons. This demands the need to identify additional critical biomarkers that correlate with intravascular leakage during dengue illness [43]. Besides, the ADE which is the key factor for severe dengue differs between individual patients. In this regard, some of the biomarkers associated with vascular permeability and ADE such as angiopoietin-2, syndecan-1, heparan sulfate, IL-6, IL-18, TNFR1 in anti-dengue IgG seropositive patients [44–46] can be incorporated into the severe-dengue diagnosis panel after scientific validation. Such a test panel in a POCT format could reduce the need for multiple hospital visits, which is especially challenging for patients living in remote areas. Results from such a panel can offer crucial information to physicians, enabling them to promptly initiate necessary interventions or refer patients to specialized hospitals as needed [43].

Different strategies are under consideration for developing effective serotype specific diagnostic tools that can indicate the severity of a subsequent dengue infection due to ADE. Recently, there has been interest in applying simple, low-cost nucleic acid amplification strategies that are alternative to conventional RT-PCR. For instance, isothermal nucleic acid amplification strategies such as loop mediated isothermal amplification (L-LAMP) and RPA have been developed for DENV serotype identification [47, 48]. In addition, use of clustered regularly interspaced short palindromic repeats (CRISPR) technology towards aiding RPA and their detection show potential scope for POCT [49, 50]. Besides, combination of different detection strategies is also a current trend in diagnostic developments. For instance, DENV serotype identification using RPA combined with lateral flow detection (LFD) have been recently

Table 1: Advantages and limitations of the different analytes and detection methods used for dengue diagnosis.

Analyte	Detection method(s)	Advantages	Limitations
Virus	Virus culture using cell line after incubation with patient sera followed by confirmation using RT-PCR	Specific confirmation of the presence of active virus infection.	Labor-intensive, time consuming, requires level 2–4 biosafety laboratory consideration. Drop in positive detection percentage after day 3 of illness [15, 19].
Neutralizing factors in patient serum	Hemagglutination inhibition test (HI)	Easy to perform. Can differentiate between primary and secondary DENV infections [21].	Requires all subtypes of a virus for testing which requires good laboratory practice conditions.
	Plaque reduction neutralization test (PRNT)	Can define the infecting serotype following a primary infection. Considered a gold standard serological test and recommended by WHO against which any new assay will need be validated [21, 34].	Labor-intensive and require good laboratory practice conditions [21].
Anti-dengue IgM	MAC-ELISA	Specific confirmation of the presence of IgM that are formed against a mixture of four dengue antigens (derived from dengue virus-infected cell culture supernatants) [56]. Sensitivity: 90 %, specificity: 98 % – compared to hemagglutination inhibition assay (HIA) standard.	False positive results due to cross reactivity with other flaviviruses [19]. Presence of non-specific reactivity in sera from patients with malaria and leptospirosis [32].
Anti-dengue IgG	ELISA	Utilizes the same antigen as MAC-ELISA and correlates with HIA. When used in combination with IgM as a ratio of IgM:IgG, it can be used to distinguish primary and secondary virus infections [57, 58].	Lacks specificity within the flavivirus serocomplex groups. Cut-off for the IgM:IgG ratio is not well defined [19].
Anti-dengue IgG & IgM	Indirect immunofluorescence test	Ability to distinguish DENV serotypes and other flaviviruses simultaneously, an advantage during concomitant epidemics [59]. Sensitivity: IgG 97 %; IgM 99 %, specificity: IgG 96 %; IgM 96 % - compared with IgG and IgM ELISA [60].	Lacks high throughput adaptation [59].
	Lateral flow immunoassay (rapid test)	Rapid, simple-to-use, low cost, does not require professional staff and expensive equipment.	Limited sensitivity, semi-quantitative [38].
Viral NS1 antigen	Antigen-capture ELISA NS1-specific IgM/IgG detection by ELISA Rapid test	Greater sensitivity and specificity over other diagnostic techniques over a broad sample age (days 0–8 of illness) [15]. Sensitivity of ELISA test greater than the sensitivity of rapid test [61]. Specificity of ELISA and rapid tests>95 % using virus isolation as the reference method [61]. Provides "point of care" diagnosis when coupled with MAC-ELISA during acute and convalescent phase of disease [21].	No differentiation between different DENV serotypes [19].
Viral nucleic acid	RT-PCR and real-time RT-PCR	Singleplex and multiplex assays that can detect single and all four serotypes, respectively, are available [62]. Able to determine viral titre at the early stage of dengue illness [63].	Positive identification drops after early stages of infection (days 1–3 of illness) [15]. Requires well trained laboratory personal, specialized instrumentation and maintenance. Likelihood of false-positive reactions due to cross-contamination with dengue virus PCR products in the laboratory [64].
	Isothermal nucleic acid-sequence based amplification assay (NASBA)	Can be used to detect all four DENV serotypes using universal primers and detect them using serotype-specific capture probes. The final product is RNA, which is far less stable than DNA, minimizing the risk of cross contamination [64]. Sensitivity: 98.5 % and specificity: 100 % compared to virus culture followed by immunofluorescence assay.	Requires well trained laboratory personal, specialized instrumentation and maintenance.
	Real-time recombinase polymerase amplification (RPA)	Fast to perform (3–15 min) and can be operated in a portable device [65].	Requires trained personnel to extract purified RNA.

developed that involves a one-step sample preparation method that inactivates DENV, which is critical for the safety of lab personnel and reducing the requirements for biosafety level 2 cabinets. The limitation of this RPA-LFD strategy is that it can detect only one serotype per tube reaction [51]. In addition, rapid tests also have a limitation of being highly subjective with respect to the interpretation of developed band color in a test window. Recently, the use of artificial intelligence in a smartphone application to scan and interpret the developed bands has been developed to minimize this uncertainty and increase confidence for patient self-testing [52].

Improvements in ELISA or RDT for a POCT or high throughput setting also require improvements in the sensitivity beyond colorimetric detection. This can be made possible by adapting them for alternative strategies such as fluorescence immunoassay (FIA) and chemiluminescence immunoassay (CLIA) owing to their wide dynamic range of detection as opposed to the 2 to 4 optical density (OD) limit in colorimetry [40]. Recently, it has been found that the performance of FIA for dengue NS1 detection is time saving compared to ELISA without any compromise in sensitivity and specificity [53]. Moreover, FIA for dengue NS1 in a cassette based rapid test also showed better performance than immunochromatography based rapid test [54]. CLIA has also recently emerged as a powerful tool in diagnostic testing. CLIA has advantages of superior sensitivity and specificity over traditional ELISA. It has multiplexing ability that allows simultaneous detection of multiple biomarkers with a lower blood volume and shorter reaction time. Moreover, it is compatible with high throughput automation that provides streamlined operation with less dependence on skilled technicians and minimum risk of human error. Studies have tested the use of CLIA for the detection of dengue antigen and have shown that it reduced the volume of blood and time required by half and cost per test reduced to one third compared to ELISA [55]. Currently, chemiluminescence based assay for dengue diagnosis has been commercially achieved for a high throughput setting. However, further developments in the use of CLIA/FIA are needed to make it adaptable for POCT use for early dengue diagnosis and identifying severe dengue cases.

Conclusions

Given the current scenario of dengue spread, demand for POCT in remote locations with high sensitivity, selectivity and ability to identify disease severity is highly sought for. In this regard, new developments viral RNA amplification strategies such as RPA-LFD and antigen/antibody detection strategies such as CLIA/FIA could set the future benchmark for the diagnosis of dengue and its severity. These strategies when adapted to widespread use are envisioned to fast-track the WHO's goal of achieving zero deaths from dengue by 2030.

Research ethics: Not applicable.
Informed consent: Not applicable.
Author contributions: E.T. and A.D.S carried out the conception, design and drafting of the paper. A.R.S carried out revising the manuscript with inputs from E.T. and A.D.S. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Use of Large Language Models, AI and Machine Learning Tools: None declared.
Conflict of interest: The authors state no conflict of interest.
Research funding: None declared.
Data availability: Not applicable.

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