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Review Article

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Molecular Detection and Genotyping of Dengue Viruses: Current Techniques and Future Prospect

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The Dengue virus is a prevalent mosquito-borne disease, and its global incidence has been steadily increasing due to the favorable environmental conditions that promote mosquito breeding, primarily influenced by rising temperatures. Bangladesh has been particularly hard-hit by an intense and ongoing outbreak, resulting in a surge of cases and fatalities. Effective management of this disease necessitates the implementation of robust public health measures, including rigorous surveillance and early diagnosis. While serological tests are commonly employed in clinical diagnosis, molecular methods hold a critical role in identifying the specific Dengue virus strain, thereby contributing to a more comprehensive understanding of disease severity. This article serves as an extensive review, delving into various molecular testing techniques employed for both surveillance and clinical diagnosis. It offers valuable insights for research and clinical laboratories engaged in the detection of Dengue virus RNA in mosquitoes, environmental samples, and clinical specimens. The methods covered encompass a spectrum of approaches, including conventional PCR, isothermal amplification, real-time RT-PCR, Sanger sequencing, and whole-genome sequencing, providing a holistic overview of the available techniques. These methods play pivotal roles in clinical diagnosis, outbreak analysis, vector surveillance, and vaccine development. Furthermore, the article underscores the importance of integrating these techniques into existing healthcare systems, emphasizing their significance in ensuring precise dengue diagnostics to enhance the efficiency of disease management. These molecular methods are indispensable tools that contribute to accurate diagnosis, enable effective outbreak investigation, facilitate vector surveillance, and support vaccine preparation, thereby enhancing the overall management and control of dengue, ultimately working toward mitigating its impact on public health.

Key words: Dengue virus, PCR, Molecular detection, Molecular Typing, Vectors, Mosquitoes

Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* family with four major serotypes, DENV1 to DENV4 [1]. The virus is transmitted by mosquitoes and the primary vectors are *Aedes aegypti* and *Aedes albopictus*. In the last few decades, the endemic area, also known as "dengue zone", has rapidly expanded across tropical regions worldwide. It affects more than 3.5 billion people and causes approximately 400 million infections and over 20,000 deaths annually [2]. Experts predict that ongoing climate change and urbanization will further extend its geographical range[3]. Furthermore, in endemic areas, the situation is aggravated by insufficient community participation in prevention measures and the excessive use of non-biodegradable products.

Around 70% of the most affected countries are Asian, and many infections are still being recorded in countries like Bangladesh, Malaysia, Vietnam, and the Philippines [4]. Being an endemic country, Bangladesh experienced the largest dengue epidemic in 2019 since its first report in 2000. Among all prevalent serotypes of dengue, DENV3 was identified as the predominant serotype [5]. During the epidemic, this outbreak also expanded to a non-endemic zone in northern Bangladesh [6]. In 2022, an unusual amount of rainfall took place and caused the second-highest annual number of dengue cases. The 2022 outbreak is unique in its scale and seasonality compared to the 2019 outbreak [7]. The study also suggested the predominance of DENV3 infection with occasional co-infection with other types [8].

The laboratory diagnosis process involves detecting various components such as the virus, viral nucleic acid, antigens or antibodies, or a combination of these methods. For the patients in the acute phase, NS1 (non-structural protein 1) antigen detection test and IgM - ELISA (enzyme-linked immunosorbent assay) are more widely used. They are easy to perform and relatively inexpensive, and the specimens required are stable at room temperature. However, there are some limitations to these tests. For NS1, its sensitivity varied by DENV stereotype, and it is not able to differentiate between serotypes [9]. For IgM – ELISA, it is known for its cross-reactivity among several other members of the *Flaviviridae* family. The recent emergence of the Zika virus has added another layer of complexity to dengue serological diagnosis [10]. In contrast, well-designed nucleic acid tests (NATs) will have added and provide a better diagnosis for acutely ill patients.

This article provides a brief review of molecular tools in dengue detection and typing. Integration and analysis of these results could also pave the road for better surveillance, clinical diagnosis and outbreak investigation.

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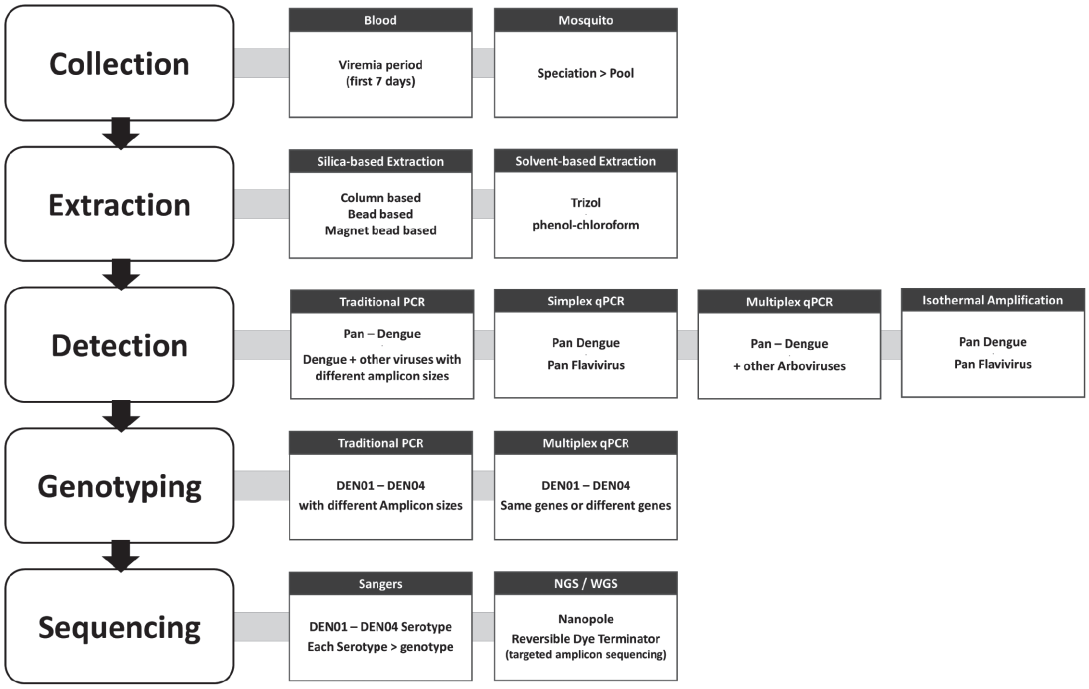

Fig. 1. *Overview of Methods and Applications for Dengue Virus Detection and Typing*

Methodologies

Pre-analytical phase

Sample collection

The success of molecular detection, such as reserve transcription polymerase chain reaction (RT-PCR), for dengue virus depends on several factors, including proper sample collection, timely sampling, proper preservation during transportation and storage, and effective sample preparation. High-quality and sufficient viral RNA extraction directly affects test sensitivity. Accurate and reproducible RT-PCR results also require proper storage of samples for downstream analysis.

Clinical samples

The best time to sample for dengue virus is when it is present in the bloodstream. However, the viremic length can vary from person to person and it can differ between primary and secondary infections [11]. In general, the suggested collection time is within the first 7 days of illness. It is also important to ensure they are stored and transported correctly to ensure the sample quality.

Serum or plasma is the most commonly used sample type for nucleic acid tests. Other sample types, such as whole blood, cerebrospinal fluid, saliva, or urine, have been used in some cases. However, their reliability for detecting dengue virus may vary depending on the stage of the infection and the viral load in the sample.

Mosquito samples

During mosquito season, Pools of up to 50 individuals of same species of mosquitoes from the same collection sites could be homogenized in 1.5 ml of BA-1 tissue culture medium and then centrifuged at 10,000 × *g* for 2 min at 4º C. A total of 140 ìl of the resulting supernatant was removed, and RNA was purified from the aliquot by using the QiaAMP viral RNA extraction kit (QIAGEN, Valencia, CA) and RNA was detected using primers recommended by the Centers for Disease Control and Prevention [12].

RNA extraction from clinical samples

Efficient procedures of extraction and purification of viral RNA from the specimen can directly impact the sensitivity and reliability of dengue virus detection. The most commonly used methods include the column-based method [13] and the Magnetic bead-based method [14] due to their quick and cost-effective characteristics.

Both methods are based on silica-based extraction. After the sample is lysed, viral RNA is exposed to silica in a solution with high ionic strength. This allows viral RNA to bind silica (which is fixed on filter membrane or magnetics beads) so it can then be washed with high salt and ethanol and then ultimately eluted with low salt [15].

If silica-based extraction is not possible, Trizol extraction and phenol-chloroform extraction are excellent alternatives. The principle of Trizol extraction is based on the use of a reagent to denature and solubilize cells and proteins, allowing for the selective extraction of RNA [16]. Phenol-chloroform extraction, on the other hand, extracts DNA and RNA using a combination of phenol and chloroform via phase separation and solvent-based denaturation [17]. Both have been used in numerous studies.

An alternative, low-cost RNA extraction method has been developed for resource-constrained settings. The method involves storing the lysed sample on an RNA-binding membrane

for over a week, eliminating the need for specialized equipment, expensive materials, and cold chain preservation [18]. This makes it easier to perform dengue testing in resource-limited areas, which is critical for early detection and effective disease management.

Analytical phase

Conventional PCR

Conventional RT-PCR based on the amplification of specific regions of the dengue virus genome using specific primers has been widely used for the detection of the dengue virus. A single-step, pan-dengue RT-PCR can detect all four DENV serotypes and only requires basic PCR and electrophoresis equipment [19]. It is a cost-effective and efficient alternative to the NS1 test for detecting dengue virus in acute-phase patients. In some studies, the PCR amplicon can also be disgusted and analyzed using restriction fragment length polymorphism (PCR-RFLP), which allows for future differentiation between the two groups of DENV (DENV1-2 and DENV3-4) [20].

A well-designed conventional RT-PCR test can detect and distinguish all four Dengue virus serotypes in a single reaction, which is more efficient than methods that require separate reactions for each serotype. To improve sensitivity and accuracy, the test employs a single 52 primer set that targets a conserved region of the capsid gene. The amplicon size for each serotype from RT-PCR is different, 482 bp (DENV1), 119 bp (DENV2), 290 bp (DENV3), and 389 bp (DENV4). It is easy to type them from a gel image [21]. This method has been improved by other group for better detection, especially for Dengue-2 and Dengue-4 viruses, as well as the Chikungunya virus [22].

There is some approach to using traditional RT-PCR for Pan Flavivirus detection. Primers were designed to target non-structural coding gene 5 (NS5), which is highly conserved among the Flavivirus. So this single tube RT-PCR assay allows to detection of many mosquito-borne *Flaviviruses* including representative strains of dengue viruses 1, 2, 3, and 4, the yellow fever virus vaccine strain, West Nile virus, Kunjin virus and St. Louis encephalitis virus [23].

The further study added one RFLP component on top of this one-step RT-PCR protocol. It can not only be used to serotype DENV 1-4 but also can distinguish among St. Louis encephalitis, West Nile, and Yellow Fever [24].

Isothermal amplification

PCR needs a thermocycler and strict reaction conditions, which raises the costs and complexity of device integration, operation, and maintenance. A low-cost alternatives like isothermal amplification can be a feasible solution. It operates at one temperature setup and does not require sophisticated equipment. This cheaper setup allows moving the molecular diagnosis from centralized labs to the front line, which can dramatically improve point-of-care diagnosis and enhance healthcare and surveillance [25]. The two most common isothermal amplification methods used are reverse transcriptase loop-mediated isothermal application (RT-LAMP) and reverse transcription recombinase polymerase amplification (RT-RPA).

Several RT-LAMP assays have been created for detecting DENV and targeting different genes, such as the C-prM gene [26], 32 UTR [27], or the non-structural protein-1 [28]. More recent designs aim to target multiple regions, such as NS2A for DENV1, NS4B for DENV2, NS4A for DENV3, and 32 UTR for DENV4, to improve specificity [29]. These RT-LAMP assays are cost-effective and reliable alternatives for clinical diagnosis and even for monitoring vector populations [30, 31]. Different methods are used to detect signals from isothermal amplification, depending on the application. Some use agarose gel electrophoresis followed by staining with ethidium bromide [32], and tube scanner [33]. Some, particularly for point-of-care setups, have moved from costly readers to direct visual reading using CYBR-Green [34] or lateral flow strips [35].

Real-time RT- PCR - Singleplex

In comparison to conventional gel-based PCR, RT-PCR has become a popular way to detect viruses in the past two decades because of its superior sensitivity and specificity. Its standard format, reproducibility, and ease of integration with fully automated systems have made it the gold standard in many laboratories [36].

Singleplex real-time RT-PCR does not need advanced thermal cyclers to manage multiple fluorophores, making it simple and more affordable to implement in laboratories. With proper primer and probe design, it can still detect all DENV serotypes. For instance, a protocol has been developed to target the 32 NC region, conserved in all DENV serotypes, in a pan-dengue singleplex RT-PCR [37, 38].

A pan-flavivirus RT-PCR assay has been developed using degenerate primers and probes to target the NS5 gene. This assay can detect 30 different Flaviviruses, including pathogenic agents like yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV) and dengue virus (DENV 1-4) due to the use of multiple possible bases in some positions of the primers and probes [39]. Even though this test cannot differentiate between different viruses, it offers wide coverage and is an effective tool for screening purposes.

Real-time RT-PCR – Multiplex

Multiplex real-time RT-PCR has the advantage of detecting and typing DENV in a single reaction. The CDC DENV1-4 RT-PCR Assay is a commonly used test in the US, being one of the few approved by the FDA for this purpose [40]. This assay consists of four reactions targeting different locations (DENV1/NS5, DENV2/E, DENV3/preM, DENV4/preM) [41] and allows for serotyping of dengue spontaneity. It can provide several benefits in understanding and managing dengue outbreaks.

Multiplex real-time RT-PCR is useful in detecting and differentiating Zika, dengue, and chikungunya viruses. These viruses are all transmitted by Aedes mosquitoes and can co-circulate and cause co-infections in the same areas. The symptoms of these diseases can be similar, making it difficult to differentiate between them in serology tests. A ZDC triplex real-time RT-PCR can detect and differentiate these three pathogens in one sample [42]. Multiplex real-time RT-PCR was developed in China to detect Zika, dengue, chikungunya, and yellow fever viruses while Zika, dengue, chikungunya, and West Nile viruses detection were included for North American use [43].

On top of pathogen detection and typing, many assays utilize the extra detection channel and incorporate quality assurance elements to ensure accurate results. For instance, the use of an RNase P detection channel serves as an internal control to monitor sample quantity, quality, and the presence of PCR inhibitors [44]. Another way of ensuring accuracy is by including a passive internal reference in the reaction to standardize fluorescence fluctuations that are not related to the PCR, such as pipetting errors and sample evaporation [45].

Sanger sequencing

The global expansion of dengue viruses has contributed to the divergence, transmission, and establishment of genetic lineages of epidemiological concerns. The dengue virus is classified into four distinct serotypes, DENV1-4, which share approximately 65% amino acid sequence similarity. Each DENV serotype includes several genotypes. Genotype is defined as a group of DENV isolates that have no more than 6% nucleotide sequence divergence. According to this definition, DENV1, DENV2, DENV3, and DENV4 can be classified into five (I, II, III, IV, and V), six (Asian I, Asian II, Cosmopolitan, American, American/Asian, and Sylvatic), four (I, II, III, and V), and four (I, II, III, and Sylvatic) genotypes, respectively [46]. Due to the diversity among serotypes, dengue virus sequencing analyses usually perform on intra-serotype antigenic variation. This is partially true for local or state-wise molecular epidemiology works.

The DENV genome, approximately 10-11 kbp in length, includes three structural genes and seven non-structural coding genes, all translated into a single polypeptide with two untranslated regions (UTRs) at both ends. Different regions of the viral genome have been chosen as study targets. The commonly used genes for dengue virus sequencing and epidemiology studies are the envelope (E) and non-structural protein 5 (NS5) genes. The E gene is conserved and large, making it useful for detecting variations among different strains [47]. On the other hand, the NS5 gene has a high mutation rate, which allows researchers to trace the evolution of the virus. These features make both E and NS5 genes valuable targets for dengue virus research and surveillance.

In recent years, the E protein coding sequence has become more popular as it offers a convenient target given the large number of E sequences available in GenBank. Furthermore, its use for genotyping has been validated in several reports, and the fragment has enough phylogenetic information to distinguish DENV from other Flaviviruses and differentiates between serotypes, genotypes, and individual strains [48]. Additionally, the phylogenetic trees for DENV reconstructed from the E coding sequence yield similar results to those constructed from full genome sequences, making genotyping more cost-effective and efficient [49].

However, despite all decades of research and modern advances in genomics, many public health laboratories in dengue-endemic regions still lack access to dengue molecular epidemiology capacity. So the CDC has created the CDC DENV E gene sequencing assay to improve surveillance and laboratory research capabilities and enhance genomic surveillance of DENV. This assay is a standard, serotype-specific method that uses Sanger sequencing and genotyping analysis of the envelope glycoprotein coding sequence (E) from clinical serum samples. This method can be utilized by surveillance and research labs to gather dengue genomic data, which will enhance the comprehension of DENV genotypes, diversity, and transmission patterns and aid in improving disease prevention measures [50].

Whole Genome Sequencing

In recent years, more high-throughput next-generation sequencing (NGS) has been used increasingly in infectious disease surveillance [51]. Two main strategies for using NGS in studying infectious diseases are metagenomic sequencing and targeted amplicon sequencing. These approaches to whole-genome sequencing have been developed and used in sequencing-based surveillance of both endemic and emerging diseases. A few next-generation sequencing methods for emerging vector-borne diseases have been developed and tested in studies of ZIKA [52], chikungunya [53], and dengue [54]. They can even be performed directly from clinical specimens with some modifications.

The two most common platforms of NGS are Illumina (reversible dye terminator sequencing) and MinION (nanopore sequencing). Both of them have been used for dengue NGS [55]. Targeted-amplicon sequencing can enhance the signals from substantial host or background genome noises [56]. The relatively small size of the DENV genome has the advantage, as we do not need that many primer sets to cover the whole genome. For previously confirmed cases, It is recommended to use this approach for both Illumina and MinION platforms.

In general, viral RNA is extracted from confirmed dengue specimens, converted to cDNA through reversed transcription, and enriched by multiple PCRs. The purified amplicons are sequenced to generate viral consensus sequences for downstream assembling. The amplification efficiency varied across amplicons, and different DENV serotypes/genotypes. In general, varied template concentrations might lead to different

coverage depths of the mapping result. However, it is less significant with the target enrichment [57].

While the precise details of whole genome sequencing are beyond the scope of this paper, it is crucial to obtain a clear and high-quality representation of the genome data from the wet lab to conduct an effective analysis of the entire genome sequence. This entails several processes such as DNA extraction, sequencing, assembly, annotation, and detection of genetic variations. The resulting data can then be applied to various fields including the discovery of new genes, functional analysis, and evolutionary studies [58].

Post-analytical phase

Traditional epidemiology employs observational or descriptive methods to uncover patterns and risk factors for diseases in populations. This includes gathering information on demographics, symptoms, location, time, and exposure to dengue vectors, as well as utilizing serology tests to minimize misdiagnosis or overdiagnosis [59]. However, there are still lots of limitations. PCR testing has improved the accuracy of data by eliminating cross-reaction issues from serology and distinguishing between current and past infections, leading to a more precise analysis of classical epidemiology [60].

Sequencing analysis of the dengue virus may require lots of computational resources and expertise in bioinformatics. However, the in-depth data obtained from sequencing provides a better understanding of viral strains. The results can reveal the relationships between the outbreak sample and samples from other locations. It enables us to identify the particular strain of the virus and track its origin and spread [61]. It also allows us to monitor any changes in dengue genotypes and lineages in specific geographic regions. This information is crucial for preventing severe outbreaks, especially in populations with no prior immunity, as seen in cases of serological naivety [62]. The analysis of the geographical origins of the outbreak highlights the importance of early detection of suspected cases, prompt implementation of control policies by local public health authorities, and reinforcing vector surveillance strategies in preventing dengue epidemics [63].

Sequence analysis can also provide information on genetic variation where a single nucleotide in the sequence differs between individuals or populations. The identification of single nucleotide polymorphisms (SNPs) allows us to identify mutations in the antigenic site and further evaluate their antigenic characteristics. This approach has been used to study the differences in antigenic evolution, especially between genotypes or during major genotype replacement events [64].

Discussion

Vector surveillance

One of the benefits of molecular tests is their ability to be conducted on mosquitoes. The arbovirus panel test has the capability to distinguish between various vector-borne viruses. Sequencing information can enhance the identification of dengue viruses that are concurrently present in both mosquitoes and humans [65]. Identifying the dengue virus temporally and spatially related to cases and vectors through molecular testing constitutes a reliable strategy for monitoring and assessing the endemic nature of the disease and facilitates the integration of surveillance data by public health officials [66].

Clinical Diagnosis

Dengue is commonly diagnosed based on clinical symptoms or serological tests. However, this can lead to a high degree of uncertainty in diagnosis. So it is crucial to improve the diagnostic infrastructure to reduce the risk of severe complications and deaths associated with this disease. RT-PCR and other molecular test methods offer high sensitivity and specificity results and are capable of confirming the specific virus and subtype. As these tests only cover the acute phase, if needed, we also need to include serology tests to assess the stage of the disease. Constant monitoring of the mutants in currently circulating strains in the primer/probe area is extremely important. Additionally, following any recent advancements in technology is important. By enhancing the accuracy of early dengue case detection using improved diagnostic infrastructure, the public health system can reduce the incidence of severe complications and fatalities related to dengue [67].

Outbreak Investigation

Managing dengue in our population has posed many challenges to us due to the highly adaptable nature of the virus [68]. Molecular tests offer several advantages in this field and are instrumental in outbreak investigation and control. By using RT-PCR, we can identify and differentiate dengue in its early stages. Additionally, the typing capacity of this test allows us to further characterize the sample by serotype, providing us with more information to easily spot patterns easier. Sequencing is the downstream test for RT-PCR, improving laboratory sample referral mechanisms for confirmation and subtyping can greatly enhance the public health system's ability to respond to dengue outbreaks [69].

Vaccine Preparation

The most effective approach to preventing and reducing vector-borne diseases is by using vaccines. Dengvaxia, a tetravalent dengue vaccine, offers protection against the dengue virus. However, it is not accessible in Bangladesh yet [70]. Nevertheless, introducing molecular diagnostic capabilities for dengue in Bangladesh can build the necessary infrastructure for the successful administration of Dengvaxia. It is important to note that this vaccine has been linked to potential side effects, especially for people who have never been exposed to the dengue virus before. Future exposure to the virus may increase the risk of severe dengue. Hence, this vaccine is only recommended for individuals who have confirmed dengue diagnosis. By

implementing molecular diagnostic tests for accurate confirmation of dengue infections, false positive results can be reduced, and only those with prior exposure to the virus will receive the vaccine. This will help minimize the risk of severe dengue outcomes [71].

Resources

The COVID-19 pandemic has resulted in the establishment of detection and surveillance infrastructure [72] that can potentially be leveraged for dengue virus detection and monitoring, given the availability of appropriate tests and the capability of these systems to meet the demands of dengue surveillance. A widespread testing and reporting approach, similar to that employed for COVID-19, can be adopted for dengue. Additionally, this infrastructure can also be used for influenza detection. In Bangladesh, where influenza and dengue seasons [73] are distinct, the same molecular detection and typing system can be employed for both viruses, as the tests and their interpretation are similar. By utilizing the same laboratory equipment and personnel, it becomes feasible to detect both influenza and dengue throughout the year.

Conclusion

This article presents an overview of the common techniques used for testing and typing the Dengue virus. These techniques are used in both well-equipped reference laboratories and resource-limited settings, and the choice of method is based on various factors such as the purpose of the test, available laboratory facilities, technical expertise, cost, time of sample collection, and transportation. The article provides a brief summary of molecular detection and typing methods, with a particular focus on their application in diagnosing acute patients and tracking the disease's spread.

To properly diagnose dengue fever, it is important to implement reliable methods that can detect the virus in patients. Among the molecular detection methods, conventional RT-PCR, isothermal amplification, and real-time RT-PCR have proven to be effective in identifying the dengue virus in patients during the acute phase of infection. These methods are more reliable than NS1 or IgM serology methods. Additionally, these molecular tests can also detect other Flaviviruses in the same reaction, making the diagnostic process more efficient and improving the accuracy of results for patients with similar symptoms. Some of these tests can also distinguish between all four dengue virus serotypes, providing more detailed information for accurate outbreak investigation and epidemiological analysis.

Our understanding of the transmission and evolution of infectious diseases has been significantly enhanced by genomic sequencing. The ability to track outbreaks, pinpoint the source of infections, and track the evolution of the pathogen is made possible by this technique's insightful insights into the genetic diversity and structure of the pathogenic agent. Public health officials can use the extensive data generated by Sanger sequencing to analyze outbreaks, make well-informed decisions, and put in place efficient control measures to stop the disease's spread. In order to provide a large volume of data quickly, whole genome sequencing, a more sophisticated and effective method, adds a new dimension to molecular epidemiology. The SARS-CoV-2 pandemic served as a test case for this method's maturity.

In conclusion, the testing and typing of the dengue virus are crucial for accurate patient diagnosis and effective disease monitoring. The advancement of molecular diagnostic methods and whole genome sequencing holds promise for the future, offering improved accuracy and the ability to respond to outbreaks effectively.

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References

1. WHO. Global Strategy for Dengue Prevention and Control, 2012–2020. WHO Press; Geneva, Switzerland: 2012.
2. Bhatt S, et al. 2013. The global distribution and burden of dengue. Nature 496, 504.
3. Messina JP, set al. 2019. The current and future global distribution and population at risk of dengue. *Nat Microbiol.* **4**(9):1508-1515.
4. World Health Organization. 2014. Dengue and Severe Dengue; World Health Organization, Regional Office for the Eastern Mediterranean: Cairo, Egypt.
5. WHO Virtual Meeting of Regional Technical Advisory Group for dengue and other arbovirus diseases (New Delhi, India, 4–6 October 2021) SEA-CD-331 World Health Organization 2022
6. Rafi A, Mousumi AN, Ahmed R, Chowdhury RH, Wadood A, Hossain G. 2020. Dengue epidemic in a non-endemic zone of Bangladesh: Clinical and laboratory profiles of patients. *PLoS Negl Trop Dis*. **14**(10):e0008567. doi: 10.1371/journal.pntd.0008567.
7. WHO Disease News - Dengue – Bangladesh 28 November 2022 https://www.who.int/emergencies/disease-outbreak-news/item/2022-DON424
8. Prattay KMR, Sarkar MR, Shafiullah AZM, Islam MS, Raihan SZ, Sharmin N. 2022. A retrospective study on the socio-demographic factors and clinical parameters of dengue disease and their effects on the clinical course and recovery of the patients in a tertiary care hospital of Bangladesh. *PLoS Negl Trop Dis.* **16**(4): e0010297. doi: 10.1371/journal.pntd.0010297.
9. Martínez-Cuellar C, Lovera D, Galeano F, Gatti L, Arbo A. 2020. Non-structural protein 1 (NS1) of dengue virus detection correlates with severity in primary but not in secondary dengue infection. *J Clin Virol*. **124**:104259. doi: 10.1016/j.jcv.2020.104259.
10. Thomas SJ, Nisalak A, Anderson KB, Libraty DH, Kalayanarooj S, Vaughn DW, Putnak R, Gibbons RV, Jarman R, Endy TP. 2009. Dengue plaque reduction neutralization test (PRNT) in primary and secondary dengue virus infections: How alterations in assay conditions impact performance. *Am J Trop Med Hyg.* **81**(5):825-33. doi: 10.4269/ajtmh.2009.08-0625.
11. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A, Titer DV, Pattern AR. 2000. Virus Serotype Correlate with Disease Severity, *The Journal of Infectious Diseases*, 181(1) 2–9, https://doi.org/10.1086/315215

12. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, Komar N, Panella NA, Allen BC, Volpe KE, et al. 2000. Rapid detection of west nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. *J Clin Microbiol.* **38**:4066–4071.

13. De Paula SO, Nunes C, Matos R, de Oliveira ZM, Lima DM, da Fonseca BA. 2001. Comparison of techniques for extracting viral RNA from isolation-negative serum for dengue diagnosis by the polymerase chain reaction. *J Virol Methods*. **98**(2):119-25. doi: 10.1016/s0166-0934(01)00371-8.

14. Patramool S, Bernard E, Hamel R, Natthanej L, Chazal N, Surasombatpattana P, Ekchariyawat P, Daoust S, Thongrungkiat S, Thomas F, Briant L, Missé D. 2013. Isolation of infectious chikungunya virus and dengue virus using anionic polymer-coated magnetic beads. *J Virol Methods.* **193**(1):55-61. doi: 10.1016/j.jviromet.2013.04.016. Epub 2013 May 10. PMID: 23669102.

15. Verheyden B, Thielemans A, Rombaut B, Kronenberger P. 2003. RNA extraction for quantitative enterovirus RT-PCR: comparison of three methods. *J Pharm Biomed Anal.* **33**(4):819-23. doi: 10.1016/s0731-7085(03)00312-1.

16. Anwar A, Wan G, Chua KB, August JT, Too HP. 2009. Evaluation of pre-analytical variables in the quantification of dengue virus by real-time polymerase chain reaction. *J Mol Diagn*. **11**(6):537-42. doi: 10.2353/jmoldx.2009.080164, PMID: 19815693.

17. Bhatnaga J, Blau D, Shieh WJ. 2012. Detection and Typing of Dengue Viruses from Archived Tissues of Fatal Cases by RT-PCR and Sequencing: Diagnostic and Epidemiologic Implications. *Am J Trop Med Molecular Hyg.* **86**(2): 335–340. doi: 10.4269/ajtmh.2012.11-0346.

18. Hernandez S, Cardozo F, Myers DR, Rojas A, Waggoner JJ. 2022. Simple and Economical Extraction of Viral RNA and Storage at Ambient Temperature. *Microbiol Spectr.* **10**(3):e0085922. doi: 10.1128/spectrum.00859-22, PMID: 35647876.

19. Sudiro TM, Ishiko H, Green S, Vaughn DW, Nisalak A, Kalayanarooj S, Rothman AL, Raengsakulrach B, Janus J, Kurane I, Ennis FA. 1997. Rapid diagnosis of dengue viremia by reverse transcriptase-polymerase chain reaction using 3'-noncoding region universal primers. *Am J Trop Med Hyg.* Apr;56(4):424-9. doi: 10.4269/ajtmh.1997.56.424.

20. Liotta DJ, Cabanne G, Campos R, Tonon SA. 2005. Molecular detection of dengue viruses in field caught Aedes aegypti mosquitoes from northeastern Argentina. *Rev Latinoam Microbiol*. **47**(3-4):82-7.

21. Harris E, Roberts TG, Smith L, Selle J, Kramer LD, Valle S, Sandoval E, Balmaseda A. 1998. Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *J Clin Microbiol*. **36**(9):2634-9. doi: 10.1128/JCM.36.9.2634-2639.1998.

22. Islam MA, El Zowalaty ME, Islam S, Sharif M, Rahman MR, Amin MR, Ali MM, Rahman MT, Morita K, Ashour HM. 2020. A Novel Multiplex RT-PCR Assay for Simultaneous Detection of Dengue and Chikungunya Viruses. *Int J Mol Sci.* **21**(21):8281. doi: 10.3390/ijms21218281.

23. Ayers M, Adachi D, Johnson G, Andonova M, Drebot M, Tellier R. 2006. A single tube RT-PCR assay for the detection of mosquito-borne flaviviruses. *J Virol Methods*. **135**(2):235-9. doi: 10.1016/j.jviromet.2006.03.009.

24. Ortiz A, Capitan Z, Mendoza Y, Cisneros J, Moreno B, Zaldivar Y, Garcia M, Smith RE, Motta J, Pascale JM. 2012. Simple, specific molecular typing of dengue virus isolates using one-step RT-PCR and restriction fragment length polymorphism. *J Virol Methods*. **185**(1):129-35. doi: 10.1016/j.jviromet.2012.06.016.

25. Obande GA, Banga Singh KK. 2020. Current and Future Perspectives on Isothermal Nucleic Acid Amplification Technologies for Diagnosing Infections. Infect Drug Resist. **13**:455-483. doi: 10.2147/IDR.S217571.

26. Lu X, Li X, Mo Z, Jin F, Wang B, Zhao H, et al. 2012. Rapid identification of Chikungunya and Dengue virus by a real-time reverse transcription-loop-mediated isothermal amplification method. *Am J Trop Med Hyg.* **87**(5):947–953. doi: 10.4269/ajtmh.2012.11-0721.

27. Teoh BT, Sam SS, Tan KK, Johari J, Danlami MB, Hooi PS, et al. 2013. Detection of dengue viruses using reverse transcription-loop-mediated isothermal amplification. *BMC Infect Dis.***13**:387. doi: 10.1186/1471-2334-13-387.

28. Hu SF, Li M, Zhong LL, Lu SM, Liu ZX, Pu JY, Wen JS, Huang X. 2015. Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1-4. *BMC Microbiol*. **15**:265. doi: 10.1186/s12866-015-0595-1.

29. Neeraja M, Lakshmi V, Lavanya V, Priyanka EN, Parida MM, Dash PK, et al. 2015. Rapid detection and differentiation of dengue virus serotypes by NS1 specific reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay in patients presenting to a tertiary care hospital in Hyderabad, India. *J Virol Methods*. 211:22–31.

doi: 10.1016/j.jviromet.2014.10.005.

30. Gaber M, Ahmad AA, El-Kady AM, Tolba M, Suzuki Y, Mohammed SM, Elossily NA. 2022. Dengue fever as a reemerging disease in upper Egypt: Diagnosis, vector surveillance and genetic diversity using RT-LAMP assay. *PLoS One.* **17**(5):e0265760.

doi: 10.1371/journal.pone.0265760.

31. Teoh BT, Sam SS, Tan KK, Danlami MB, Shu MH, Johari J, Hooi PS, Brooks D, Piepenburg O, Nentwich O, Wilder-Smith A, Franco L, Tenorio A, AbuBakar S. 2015. Early detection of dengue virus by use of reverse transcription-recombinase polymerase amplification. *J Clin Microbiol*. **53**(3):830-7. doi: 10.1128/JCM.02648-14.

32. Xiong, Y., Luo, Y., Li, H., Wu, W., Ruan, X., and Mu, X. 2020. Rapid Visual Detection of Dengue Virus by Combining Reverse Transcription Recombinase-Aided Amplification with Lateral-Flow Dipstick Assay. *Int. J. Infect. Dis.* **95**:406–412. doi:10.1016/j.ijid.2020.03.075.

33. Abd El Wahed A, Patel P, Faye O, Thaloengsok S, Heidenreich D, Matangkasombut P, Manopwisedjaroen K, Sakuntabhai A, Sall AA, Hufert FT, Weidmann M. 2015. Recombinase Polymerase Amplification Assay for Rapid Diagnostics of Dengue Infection. *PLoS One.* **10**(6):e0129682. doi: 10.1371/journal.pone.0129682.

34. Hu SF, Li M, Zhong LL, Lu SM, Liu ZX, Pu JY, Wen JS, Huang X. 2015. Development of reverse-transcription loop-mediated isothermal amplification assay for rapid detection and differentiation of dengue virus serotypes 1-4. *BMC Microbiol*. **15**:265. doi: 10.1186/s12866-015-0595-1.

35. Ahmed M, Pollak NM, Hugo LE, van den Hurk AF, Hobson-Peters J, Macdonald J. 2022. Rapid molecular assays for the detection of the four dengue viruses in infected mosquitoes. *Gates Open Res.* **6**:81. doi: 10.12688/gatesopenres.13534.1.

36. Boonham N, Kreuze J, Winter S, van der Vlugt R, Bergervoet J, Tomlinson J, et al. 2014. Methods in virus diagnostics: from ELISA to next generation sequencing. *Virus Res.* **186**:20–31. doi: 10.1016/j.virusres.2013.12.007.

37. Leparc-Goffart I, Baragatti M, Temmam S, Tuiskunen A, Moureau G, Charrel R, de Lamballerie X. 2009. Development and validation of real-time one-step reverse transcription-PCR for the detection and typing of dengue viruses. *J Clin Virol.* **45**(1):61–6.

38. Alm E, Lesko B, Lindegren G, Ahlm C, Söderholm S, Falk KI, Lagerqvist N. 2014. Universal single-probe RT-PCR assay for diagnosis of dengue virus infections. *PLoS Negl Trop Dis*. **8**(12):e3416. doi: 10.1371/journal.pntd.0003416.

39. Patel P, Landt O, Kaiser M, Faye O, Koppe T, Lass U, Sall AA, Niedrig M. 2013. Development of one-step quantitative reverse

transcription PCR for the rapid detection of flaviviruses. *Virol J.* **10**:58. doi: 10.1186/1743-422X-10-58.

40. Diseases (NCEZID), Division of Vector-Borne Diseases (DVBD). 2019. US Centers for Disease Control and Prevention. Molecular Tests for Dengue Virus - CDC DENV-1-4 rRT-PCR Multiplex and Trioplex rRT-PCR Assays. https://www.cdc.gov/dengue/healthcare-providers/testing/molecular-tests/index.html.

41. Santiago GA, Vergne E, Quiles Y, Cosme J, Vazquez J, et al. 2013. Analytical and Clinical Performance of the CDC Real Time RT-PCR Assay for Detection and Typing of Dengue Virus. *PLoS Negl Trop Dis.* **7**(7): e2311. doi:10.1371/journal.pntd.0002311.

42. J.J. Waggoner, L. Gresh, A. Mohamed-Hadley, G. Ballesteros, M.J. Davila, Y. Tellez, M.K. Sahoo, A. Balmaseda, E. Harris, B.A. Pinsky. 2016. Single-reaction multiplex reverse transcription PCR for detection of Zika, chikungunya, and dengue viruses. *Emerg. Infect. Dis.* **22**(7) 1295-1297. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4918162/

43. Wu W, Wang J, Yu N, Yan J, Zhuo Z, Chen M, Su X, Fang M, He S, Zhang S, Zhang Y, Ge S, Xia N. 2018. Development of multiplex real-time reverse-transcriptase polymerase chain reaction assay for simultaneous detection of Zika, dengue, yellow fever, and chikungunya viruses in a single tube. *J Med Virol.* **90**(11):1681-1686. doi: 10.1002/jmv.25253. Epub 2018 Aug 16.

44. Mishra N, Ng J, Rakeman JL, Perry MJ, Centurioni DA, Dean AB, Price A, Thakkar R, Angus AG, Williamson P, Delwart E, Carrington C, Sahadeo N, Che X, Briese T, Tokarz R, Lipkin WI. 2019. One-step pentaplex real-time polymerase chain reaction assay for detection of zika, dengue, chikungunya, West nile viruses and a human housekeeping gene. *J Clin Virol.* **120**:44-50. doi: 10.1016/j.jcv.2019.08.011.

45. Fernandes-Monteiro AG, Trindade GF, Yamamura AM, Moreira OC, de Paula VS, Duarte AC, Britto C, Lima SM. 2015. New approaches for the standardization and validation of a real-time qPCR assay using TaqMan probes for quantification of yellow fever virus on clinical samples with high quality parameters. *Hum Vaccin Immunother.* **11**(7):1865-71.

doi: 10.4161/21645515.2014.990854.

46. Thermo Fisher Scientific Inc. , ROX passive reference dye for troubleshooting real-time PCR, Application Note (ROX dye), https://assets.thermofisher.com/TFS-Assets/GSD/Application-Notes/co016741-rox-dye-for-qqpcr-app-note.pdf.

47. Harapan H, Michie A, Sasmono RT, Imrie A. 2020. Dengue: A Minireview. Viruses. **12**(8):829. doi: 10.3390/v12080829.

48. Green ED, Watson JD, Collins FS. 2015. Human Genome Project: Twenty-five years of big biology. Nature. **526**(7571):29-31. doi: 10.1038/526029a.

49. Zhang X, Jia R, Shen H, Wang M, Yin Z, Cheng A. 2017. Structures and Functions of the Envelope Glycoprotein in Flavivirus Infections. Viruses. **9**(11):338. doi: 10.3390/v9110338.

50. Rodríguez-Aguilar ED, Martínez-Barnetche J, Juárez-Palma L, Alvarado-Delgado A, González-Bonilla CR, Rodríguez MH. 2022. Genetic diversity and spatiotemporal dynamics of DENV-1 and DENV-2 infections during the 2012-2013 outbreak in Mexico. Virology. **573**:141-150. doi: 10.1016/j.virol.2022.06.011.

51. Koo C, Tien WP, Xu H, Ong J, Rajarethinam J, Lai YL, Ng LC, Hapuarachchi HC. 2018. Highly Selective Transmission Success of Dengue Virus Type 1 Lineages in a Dynamic Virus Population: An Evolutionary and Fitness Perspective. iScience. **6**:38-51. doi: 10.1016/j.isci.2018.07.008.

52. Santiago GA, González GL, Cruz-López F, Muñoz-Jordan JL. 2019. Development of a Standardized Sanger-Based Method for Partial Sequencing and Genotyping of Dengue Viruses. *J Clin Microbiol.* **57**(4):e01957-18. doi: 10.1128/JCM.01957-18.

53. Maljkovic BI, Melendrez MC, Bishop-Lilly KA, Rutvisuttinunt W, Pollett S, Talundzic E, Morton L, Jarman RG. 2020. Next generation sequencing and bioinformatics methodologies for infectious disease research and public health: approaches, applications, and considerations for development of laboratory capacity. J Infect Dis **221**:S292–S307.

54. Chen Z, Azman AS, Chen X, Zou J, Tian Y, Sun R, Xu X, Wu Y, Lu W, Ge S, Zhao Z, Yang J, Leung DT, Domman DB, Yu H. 2022. Global landscape of SARS-CoV-2 genomic surveillance and data sharing. *Nat Genet.* **54**(4):499-507. doi: 10.1038/s41588-022-01033-y.

55. Nakayama E, Tajima S, Kotaki A, Shibasaki KI, Itokawa K, Kato K, Yamashita A, Sekizuka T, Kuroda M, Tomita T, Saijo M, Takasaki T. 2018. A summary of the imported cases of Chikungunya fever in Japan from 2006 to June 2016. *J Travel Med.* **25**(1):1-11. doi: 10.1093/jtm/tax072.

56. Aw PP, de Sessions PF, Wilm A, Hoang LT, Nagarajan N, Sessions OM, Hibberd ML. 2014. Next-generation whole genome sequencing of dengue virus. *Methods Mol Biol*. 1138:175-95. doi: 10.1007/978-1-4939-0348-1_12.

57. Kafetzopoulou LE, Efthymiadis K, Lewandowski K, Crook A, Carter D, Osborne J, Aarons E, Hewson R, Hiscox JA, Carroll MW, Vipond R, Pullan ST. 2018. Assessment of metagenomic Nanopore and Illumina sequencing for recovering whole genome sequences of chikungunya and dengue viruses directly from clinical samples. Euro Surveill 23:1800228.

58. Quick J, Grubaugh ND, Pullan ST, et. al. . 2017. Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples. Nat Protoc **12**:1261–1276.

59. Su W, Jiang L, Lu W, Xie H, Cao Y, Di B, Li Y, Nie K, Wang H, Zhang Z, Xu S. 2022. A Serotype-Specific and Multiplex PCR Method for Whole-Genome Sequencing of Dengue Virus Directly from Clinical Samples. Microbiol Spectr. **10**(5): e0121022. doi: 10.1128/spectrum.01210-22. Epub 2022 Sep 12. PMID: 36094197;

60. Dang TT, Pham MH, Bui HV, et al. 2020. Whole genome sequencing and genetic variations in several dengue virus type 1 strains from unusual dengue epidemic of 2017 in Vietnam. *Virol J* **17**(7). https://doi.org/10.1186/s12985-020-1280-z.

61. Raafat N, Blacksell SD, Maude RJ. 2019. A review of dengue diagnostics and implications for surveillance and control. *Trans R Soc Trop Med Hyg*. **113**(11):653-660. doi: 10.1093/trstmh/trz068.

62. Lim JK, Seydou Y, Carabali M, Barro A, Dahourou DL, Lee KS, Nikiema T, Namkung S, Lee JS, Shin MY, Bonnet E, Kagone T, Kaba L, Edwards T, Somé PA, Yang JS, Alexander N, Yoon IK, Ridde V. 2019. Clinical and epidemiologic characteristics associated with dengue during and outside the 2016 outbreak identified in health facility-based surveillance in Ouagadougou, Burkina Faso. *PLoS Negl Trop Dis*. **13**(12):e0007882. doi: 10.1371/journal.pntd.0007882.

63. Guo C, Zhou Z, Wen Z, Liu Y, Zeng C, Xiao D, Ou M, Han Y, Huang S, Liu D, Ye X, Zou X, Wu J, Wang H, Zeng EY, Jing C, Yang G. 2017. Global Epidemiology of Dengue Outbreaks in 1990-2015: A Systematic Review and Meta-Analysis. *Front Cell Infect Microbiol.* **7**:317. doi: 10.3389/fcimb.2017.00317.

64. Choudhury MA, Lott WB, Aaskov J. Distribution of fitness in populations of dengue viruses. PLoS One. 2014 Sep 15;9(9):e107264. doi: 10.1371/journal.pone.0107264.

65. Sun J, Zhang H, Tan Q, Zhou H, Guan D, Zhang X, Duan J, Cai S, Peng Z, He J, Ke C, Lin J, Liu T, Ma W, Wu D. 2018. The epidemiological characteristics and molecular phylogeny of the dengue virus in Guangdong, China, 2015. *Sci Rep.* **8**(1):9976. doi: 10.1038/s41598-018-28349-2.

66. Katzelnick LC, Coello Escoto A, Huang AT, Garcia-Carreras B, Chowdhury N, Maljkovic Berry I, Chavez C, Buchy P, Duong V, Dussart P, Gromowski G, Macareo L, Thaisomboonsuk B, Fernandez S, Smith DJ, Jarman R, Whitehead SS, Salje H, Cummings DAT.

2021. Antigenic evolution of dengue viruses over 20 years. Science. **374**(6570):999-1004. doi: 10.1126/science.abk0058.

67. Garza CE, et al. 2014. Detection of Aedes aegypti mosquitoes infected with dengue virus as a complementary method for increasing the sensitivity surveillance: identification of serotypes 1, 2, and 4 by RT-PCR in Quintana Roo, Mexico. Southwest Entomol.39:307–316.

68. da Silva SJR, do Nascimento JCF, Germano Mendes RP, Guarines KM, Targino Alves da Silva C, da Silva PG, de Magalhães JJF, Vigar JRJ, Silva-Júnior A, Kohl A, Pardee K, Pena L. 2022. Two Years into the COVID-19 Pandemic: Lessons Learned. *ACS Infect Dis.* **8**(9):1758-1814. doi: 10.1021/acsinfecdis.2c00204.

69. Kok BH, Lim HT, Lim CP, Lai NS, Leow CY, Leow CH. 2023. Dengue virus infection - a review of pathogenesis, vaccines, diagnosis and therapy. Virus Res. 324:199018. doi: 10.1016/j.virusres.2022.199018.

70. Srisuphanunt M, Puttaruk P, Kooltheat N, Katzenmeier G, Wilairatana P. 2022. Prognostic Indicators for the Early Prediction of Severe Dengue Infection: A Retrospective Study in a University Hospital in Thailand. *Trop Med Infect Dis.* **7**(8):162. doi: 10.3390/tropicalmed7080162. Erratum in: Trop Med Infect Dis. **7**(11).

71. Liu L, Wu T, Liu B, Nelly RMJ, Fu Y, Kang X, Chen C, Huang Z, Wu B, Wang J, Zhu Z, Ma J, Liu M, Zhang Y, Bao C, Lin F, Chen W and Xia Q. 2021. The Origin and Molecular Epidemiology of Dengue Fever in Hainan Province, China, 2019. *Front. Microbiol.* 12:657966. doi: 10.3389/fmicb.2021.657966.

72. Raafat N, Blacksell SD, Maude RJ. 2019. A review of dengue diagnostics and implications for surveillance and control. *Trans R Soc Trop Med Hyg.* **113**(11):653-660. doi: 10.1093/trstmh/trz068.

73. Dengue vaccine: WHO position paper, September 2018 - Recommendations. *Vaccine.* 2019 Aug 14; **37**(35):4848-4849. doi: 10.1016/j.vaccine.2018.09.063.