Goncalves, Adriana; Peeling, Rosanna W; Chu, May C; Gubler, Duane J; de Silva, Aravinda M; Harris, Eva; Murtagh, Maurine; Chua, Arlene; Rodriguez, William; Kelly, Cassandra; +1 more... Wilder-Smith, Annelies; (2017) Innovative and New Approaches to Laboratory Diagnosis of Zika and Dengue: A Meeting Report. The Journal of infectious diseases, 217 (7). pp. 1060-1068. ISSN 0022-1899 DOI: https://doi.org/10.1093/infdis/jix678

Downloaded from: http://researchonline.lshtm.ac.uk/id/eprint/4645959/

DOI: https://doi.org/10.1093/infdis/jix678

Usage Guidelines:

Please refer to usage guidelines at https://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by-nc-nd/2.5/
Innovative and new approaches to laboratory diagnosis of Zika and dengue: a meeting report

Running Title: Innovative diagnostic approaches for flaviviruses

Brief summary: Novel diagnostic approaches for Zika and dengue are on the rise but may not make it to the market due to bottlenecks in access to samples for validation. An international reference laboratory response is needed to address these challenges which include networks of in-country laboratories, with well-characterized samples to facilitate assay validation and ensure quality control.

Adriana Goncalves¹, Rosanna W. Peeling¹, May C. Chu², Duane J. Gubler³, Aravinda M. de Silva⁴, Eva Harris⁵, Maurine Murtagh⁶, Arlene Chua⁷, William Rodriguez⁸, Cassandra Kelly⁹, Annelies Wilder-Smith⁹,¹⁰,¹¹

¹ London School of Hygiene & Tropical Medicine, London, UK
² Department of Epidemiology, Colorado School of Public Health, University of Colorado, Anschutz Medical Center, Aurora, Colorado, USA
³ Emerging Infectious Diseases Program, Duke-NUS Medical School, Singapore
⁴ Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC, USA
⁵ Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, CA, USA
⁶ The Murtagh Group, LLC
⁷ World Health Organization, Geneva, Switzerland
⁸ Foundation for Innovative New Diagnostics (FIND), Geneva, Switzerland
⁹ Institute of Public Health, University of Heidelberg, Germany
¹⁰ Lee Kong Chian School of Medicine, Singapore
¹¹ Department of Global Health and Epidemiology, University of Umea, Umea, Sweden

‡ Corresponding Author: Annelies Wilder-Smith.
Email: anneliesws@gmail.com

© The Author(s) 2017. Published by Oxford University Press for the Infectious Diseases Society of America. All rights reserved. For permissions, e-mail: journals.permissions@oup.com.
ABSTRACT

Epidemics of dengue, Zika, and other arboviral diseases are increasing in frequency and severity. Current efforts to rapidly identify and manage these epidemics are limited by the short diagnostic window in acute infection, the extensive serologic cross-reactivity among flaviviruses, and the lack of point-of-care diagnostics to detect these viral species in primary care settings. The Partnership for Dengue Control organized a workshop to review the current landscape of Flavivirus diagnostics, identified current gaps, and developed strategies to accelerate the adoption of promising novel technologies into national programmes. The rate-limiting step to bringing new diagnostics tools to the market is access to reference materials and well-characterized clinical samples to facilitate performance evaluation. We suggest the creation of an international laboratory response consortium for flaviviruses with a de-centralized biobank of well-characterized samples to facilitate assay validation. Access to proficiency panels are needed to ensure quality control, in additional to in-country capacity building.

Key words: Dengue, Zika, Flavivirus, Arbovirus, Laboratory, Diagnostics, Serology, Surveillance
Background

Zika virus (ZIKV) and the dengue viruses (DENV) are arthropod-borne viruses (arboviruses) of the Flaviviridae family that co-circulate in tropics and subtropics, along with other arboviruses that share the same Aedes spp. mosquito vectors [1]. Several factors including viral evolution, re-distribution of vectors, ineffective vector control strategies, population growth, urbanization, and globalization have contributed to the global spread of DENV, ZIKV and other arboviruses [2].

Up to 400 million DENV infections are estimated to occur every year [3], and infection with any of the four DENV serotypes (DENV1-4) can cause severe and sometimes fatal disease. The geographical expansion of dengue is increasingly associated with more severe disease outcomes [2, 4]. ZIKV is following the global spread of DENV [2]. ZIKV infections were first thought to only cause sporadic and mild disease in parts of Africa and Asia [5]. A major Zika outbreak with a high attack rate occurred for the first time in 2007. During a subsequent outbreak in the Pacific (French Polynesia) in 2013, ZIKV was linked to severe neurological disease in humans [6]. The recent explosive outbreak in the Americas unmasked the association between prenatal ZIKV infections and severe birth defects [2, 6].

No specific therapeutic options exist for DENV or ZIKV infections. For DENV, a vaccine was recently licensed but has not yet been implemented widely in any of the affected countries[7]; for ZIKV, at least 45 vaccine candidates are now in development but a licensed vaccine will not be available for years to come[8]. There is an urgent need for highly specific diagnostic assays that can identify and discriminate between co-circulating DENV and ZIKV for efficient case management, surveillance, control, and vaccine trials. In May 2017, the Partnership for Dengue Control (PDC)[9] organised a workshop with approximately 80 key stakeholders and thought leaders to address critical issues related to the diagnosis and surveillance of ZIKV and DENV. The workshop was organised around three questions:

i) What is the status of Zika and dengue diagnostics?
ii) What technological innovations might be available in the near, intermediate and long-term future?
iii) What is needed to make these technologies readily available where they are most needed?
The following is a summary of key outcomes that emerged from the meeting.

**What is the status of Zika and dengue diagnostics?**

Individuals infected with DENV and ZIKV may be asymptomatic or display a similar constellation of initial clinical symptoms [10]. Hence, virus-specific assays are required for accurate diagnosis. Since the first isolation of DENV during World War II [11, 12], a number of diagnostic methods commonly used for viral detection, such as viral isolation, Plaque Reduction Neutralization Test (PRNT), the IgM Enzyme-Linked Immunosorbent Assay (ELISA), and, in the 1990s, Reverse Transcription-Polymerase Chain Reaction (RT-PCR) [13] were developed for DENV (Figure 1) and other medically relevant flaviviruses.

Assays to detect DENV and ZIKV can be divided into two main categories: a) assays to detect the pathogen (viral isolation, viral nucleic-acid testing (NAT), or viral antigen detection); and b) assays to detect exposure to the pathogen (detection of virus-specific antibodies such as IgM, IgG, and IgA). Assay selection depends both on the timing of sample collection and the purpose of testing (Figure 2). The viremic period of flaviviral infections is transient and short-lived; duration of viral shedding and the presence of ZIKV RNA can be variable across sample types (e.g., serum, whole blood, urine, saliva, amniotic fluid) [6, 14] and different hosts (e.g., pregnant women, other adults) [15]. A negative viral isolation and/or NAT result does not exclude the presence of a current infection.

In the convalescent phase of infection, serologic methods are preferred, though paired acute and convalescent samples are required to distinguish current from past infections [16]. The major challenge of ZIKV and DENV diagnosis by serology is the extensive cross-reactivity of antibody responses resulting from prior flaviviral infections and/or vaccination [17-19]. Figure 3 details the applications, advantages, and limitations of the different types of assays available for the detection of DENV and ZIKV infections.

**Landscape overview and existing gaps**

Both “in-house” assays as well as commercial kits are available to detect ZIKV and DENV infections (Figure 4). Most of the available technologies require laboratory facilities with appropriate diagnostic competence (Figure 4); point-of-care assays remain limited. Zika commercial kits include NAT and serological assays. The current ZIKV NAT assays have not yet gone through much rigorous
evaluations[20], and the evidence is even scarcer for serological assays. Antigen detection assays for the diagnosis of ZIKV infections are currently not available on the market.

Performance of commercial dengue diagnostics has improved over the last decade. These include two FDA approved assays (one RT-PCR and one IgM capture ELISA). Additionally, there are several rapid lateral flow assays (RDTs) for the detection of DENV NS1 antigen, DENV-specific IgM antibodies, or both (Figure 4), none of which are FDA approved. RDTs hold promise as future point-of-care (POC) applications; however, the clinical performance of these assays has been highly variable [21].

While dengue serological assays have been clinically validated, their specificity has decreased by cross-reactivity in the context of the recent co-circulation of ZIKV [22]. In general, to date very few dengue and Zika diagnostic assays have been adequately and independently evaluated using clinical specimens from both ZIKV-infected and DENV-infected populations. Diagnostic assays that can accurately detect and, distinguish co-circulating flaviviral infections and predict severe disease outcomes at or near POC are urgently needed.

What technological innovations might be available in the near, intermediate and long-term future?

Different companies and research groups were invited to present technologies to detect DENV and ZIKV. In the following, we discuss the technologies in the pipeline (Figure 4, in bold) and their potential to change the paradigm of flaviviral diagnosis.

**Pipeline assays to detect the pathogen**

* Nucleic-acid testing (NAT)*

Simpler and faster alternatives to traditional RT-PCR methods have the potential to be used at or near POC. These include cartridge-based “sample in, answer out” multiplex real-time RT-PCR assays that can simultaneously detect ZIKV, DENV1-4, and also other arboviral infections such as chikungunya virus (CHIKV)- an alphavirus- , and other viral infections (3-plex to 6-plex combinations) from a single specimen in less than two hours. Arboviral assays are being developed for use on existing industry platforms that were previously validated and implemented for other molecular tests. This strategy illustrates the utility of “open-platform” systems that can easily incorporate
newly-developed molecular amplification methods to suit an emergent medical need, such as ZIKV. Another advantage of these systems is the ability to transmit data wirelessly and monitor the results remotely. The disadvantage is that these platforms are costly, and some require technical expertise and laboratory infrastructure that are not widely available.

The development of more portable molecular platforms linked to faster isothermal amplification methods independent of thermal cycling, such as recombinase polymerase amplification (RPA) [23], loop-mediated isothermal amplification (LAMP), and others are also underway for singleplex and multiplex detection of ZIKV and other arboviruses. In prototype formats, results can be achieved in less than one hour, and assays can potentially be applied to settings without electricity or highly trained users. Proof-of-principle studies exist [23-30], but further simplification of sample preparation and more robust clinical performance evaluation will be required. Innovative technologies, such as clustered regularly interspaced short palindromic repeats (CRISPR)-based RNA sensing, robotics, microfluidics, smartphones, and 3D printers are being used to develop these assays. Other NAT innovations include the use of paper-based strips for multiplex detection of ZIKV, DENV, and CHIKV end-point RT-PCR products [31].

Antigen detection assays

High-affinity monoclonal antibodies (Mabs) that recognize specific epitopes on ZIKV antigens are required to develop antigen detection assays, and are either in development or were developed for NS1 [32, 33], including the development of RDTs [34].

Pipeline assays to detect past exposure

Given our understanding of the cross-reactivity of current antibody detection methods for flaviviruses, there is a lack of reliable reference diagnostics against which to compare newly developed specific serological assays. Detection of virus-specific neutralizing antibodies by PRNT can be useful to discriminate viral species and serotypes in primary infections. However, the specificity of PRNT in sequential DENV infections, or sequential DENV and ZIKV infections and at early time-points post-infection is limited [32, 35, 36]. Interestingly, little cross-neutralization is detected in late convalescent samples (>2 months post-infection) after DENV and ZIKV infections [37]. These observations highlight the importance of the timing of sample collection and the history of exposure to past infections to inform the serodiagnosis of flaviviral infections. It is critical to evaluate multiple flaviviruses simultaneously in neutralization assays to interpret the results appropriately.
Strongly neutralizing human monoclonal antibodies target quaternary structure epitopes that typically bind across envelope (E) proteins displayed on the surface of the viral particles [38-40]. Epitopes with high sequence homology among serotypes and viral species can trigger cross-reactive (CR) antibody responses, whereas unique epitopes can lead to type-specific (TS) antibody responses. This information is being used to rationally design ZIKV and DENV E and NS1 recombinant antigens for specific serological assays.

Isolated human ZIKV TS anti-NS1 Mabs were used to identify TS recognition sites on ZIKV NS1 protein by antibody competition assays [32]. One of these antibodies was adapted to a competition-based ELISA in which serum antibodies are measured for their ability to block the binding of a ZIKV NS1-specific Mab to solid-phase ZIKV NS1 [41]. This approach, named ZIKV NS1 blockade-of-binding (BOB) ELISA, was shown to be more specific than traditional ELISA assays. Clinical validation in large multicentre cohorts of patients stratified by exposure to DENV and ZIKV infection, immune status and timing of sample collection confirmed the high specificity and sensitivity of the assay [41]. The ZIKV NS1 BOB ELISA has been implemented in laboratories of six different countries (Brazil, Italy, Nicaragua, Switzerland, United Kingdom, and United States).

Nanotechnology-based technologies have also been developed, including simple-to-use readout platforms with data connectivity that use disposable microfluidic cartridges for rapid detection of ZIKV and DENV antibodies/antigen; and a multiplex serological assay that uses near-infrared fluorescence enhanced (NIR-FE) imaging on a nanoscale plasmonic gold microarray antigen platform (12-plex) for antibody detection on two different channels [42]. The latter was shown to detect and distinguish IgG antibodies from ZIKV- and DENV-infected patients, as well as determine the timing of exposure to infection by measuring IgG avidity.

What is needed to make these technologies field-available?

For the last 25 years, routine diagnostic approaches have mainly included laboratory-based RT-PCR, IgM detection, and PRNT. Recognition of Zika as a public health emergency of international concern (PHEIC) has galvanized the development of new diagnostic assays to detect flaviviral infections. While these efforts must be encouraged, it is equally important to look downstream and identify the issues around translating research into a product that is field-available, robust, easy-to-use, reasonably inexpensive, accurate and has demonstrable clinical impact. Previous R&D experience has shown that the path from diagnostic development to adoption is long and fragmented [16].
There is a massive attrition from the number of tests that undergo initial development to being ultimately adopted for routine use, the so-called “valley of death”.

**How can we accelerate the pathway from discovery to adoption?**

The five major steps identified for optimization (Figure 4 A-E) are discussed below and summarized in Table 1.

**A. Market uncertainty**

For diagnostic countermeasures to be readily available, research and development (R&D) must happen before rather than in response to an outbreak [43]. The unpredictable and episodic nature of outbreaks brings uncertainty to the market, and diagnostic companies are left unable to adequately forecast demand and establish business models that allow a return on investment. Even when a product is brought into the market, there is no guarantee that it will be adopted by national health authorities. Once a PHEIC has ended, sustained manufacturing support of the product may be at stake. Sustainable markets are required to ensure that validated, approved, high-quality diagnostics remain available for use in the next outbreak event. As such, innovative financial incentives are needed to achieve sustainable emergency preparedness for diagnostics. From investments in product development to the establishment of partnerships and the creation of models to support scalable adoption into national programmes, a variety of mechanisms have been proposed or established to overcome some of the challenges.

The WHO R&D Blueprint for Actions to Prevent Epidemics has initiated a call for open-platform technologies to improve R&D preparedness against global health emergencies, so that in the event of an epidemic diagnostic kits can be made available in a short time-frame [44]. Furthermore, there was a call for a coalition between diagnostic preparedness efforts and programmes that finance and manage the development of vaccines [43]. As a result, CEPI·dx, a new partnering model between the Coalition for Epidemic Preparedness Innovations (CEPI), the Foundation for Innovative New Diagnostics (FIND) and other diagnostic partners has been created.

**B. Target Product Profiles (TPPs)**

TPPs are used to define the desired technical and operational minimum characteristics of diagnostic tests to ensure the development of the most impactful products. TPPs are aspirational in nature;
however, excessively stringent requirements may deter industry partners from developing new products and lead to a lack of diagnostic tests meeting those requirements. Strategies on how to best define the desired characteristics of TPPs and/or inform the use of diagnostic tests when those requirements are not met have been proposed. For example, a slightly less accurate test might provide a higher public health impact in terms of increased access to testing compared to a more accurate but expensive or complex test (e.g., the approach used to approve the use of HIV self-testing and malaria RDTs in the past). As such, it is important to consider how the assay will be used, in which setting, and for what purpose, e.g., surveillance, early-warning, clinical management at point of care, as different applications will have different technical and operational requirements. A weighted risk and benefits approach within different use scenarios may be more appropriate not only to define but also to guide regulatory approval and adoption.

C. Assay optimization and clinical validation

Internationally accepted reference preparations to compare and potentially standardize the different assays are crucially important[45]. WHO has established numerous reference preparations, most of them as WHO International Standards (WHO IS). For Zika RNA, the biological standard for molecular tests was characterized for the majority of NAT based assays available[46], and the complete sequence of the Zika virus of this reference preparation was published[47] and established as WHO IS. Lack of access to biobanks of well-characterized clinical specimens delays the process of test optimization, clinical validation, and product adoption. This lack was identified as the most significant bottleneck along the pathway from development to adoption.

Of note, the pathway to adoption of “in-house” assays and of commercial kits differs substantially. Quality-assured clinical laboratories can develop, validate, and then implement their “in-house” assays. In contrast, commercial diagnostic kits go through regulatory approval processes that may require large clinical validation studies, manufacturing under a quality management system, and some level of distribution capacity. The different streams of test development make it challenging to determine relative comparability of the accuracy of the different tests as very few of them share the same calibration controls (i.e., internal positive controls used for measuring the reactivity of a diagnostic test) or screening panels (i.e., a small set of coded samples that include high positive, low positive, cross-reactive, and negative samples, to measure the specificity and sensitivity of a diagnostic test). Obtaining irreplaceable clinical specimens is costly; the same test materials cannot be used throughout the development process. Access to clinical samples becomes even more
challenging during an outbreak with multiple demands to prioritize assay validation in a short time-frame and inability to do head-to-head comparisons.

A coordinated network of quality-assured laboratories that are well trained in assay validation and performance evaluation could be leveraged *a priori*. Such an approach would alleviate pressure on the countries involved in outbreak response, yet provide access to clinical samples and data in a way that may be acceptable to the different parties. Of note, local restrictions on the export of clinical samples (as witnessed during the Zika outbreak) limits sample sharing for product validation outside the affected countries [43]. The involvement of a network of capable local laboratories would have the advantage to overcome the need for out-of-country sample transfer and facilitate country involvement and capacity development at an early stage of product development. A transparent and fair process of engagement needs to be put into place to minimize distrust and ensure access and equitable sharing of specimens and data. The creation of a governance system to provide access to reference panels and protocols for test validation has been proposed.

**D. Regulatory approval**

Regulation is essential to ensure the safety, quality, and effectiveness of diagnostic tests, yet over 50% of countries do not independently regulate *in vitro* diagnostics (IVDs) [48]. The regulatory landscape for IVDs is highly variable, and regulatory approval mechanisms vary from country to country. This makes assay uptake processes slow, costly and not transparent. Regulatory harmonization between international and national regulatory agencies, coupled with coordinated information sharing among the different interest groups (industry, regulators, researchers, laboratories, health systems, and patients) is required.

During outbreak events, emergency use authorizations are generally employed to provide regulatory oversight for diagnostics that have not previously been evaluated and yet are urgently needed for global response. Both FDA (EUA) and WHO (EUAL) have implemented programs to address the evaluation of new diagnostics in an emergency setting. It is important to note that in both cases, EUA and/or EUAL approval does not extend approval for use outside of an emergency setting. Once an emergency is ended, industry will need to seek approval for regular use of their products in the intended settings using either FDA 510(k) or WHO prequalification procedures. The data obtained during EUA and/or EUAL approval may be included in the application package for regular approval; however, it is likely that additional data will be required for full approval. In these instances, it can
be challenging for industry to provide sufficient data, as limited access to well-characterized samples can prevent evaluation of the products to the extent required for FDA and WHO approval. As of May 2017, several ZIKV diagnostic assays have received EUA (15 assays) and/or EUAL (2 assays) [45]; however, no single ZIKV assay has been cleared by the FDA to date (Figure 4).

E. Sustainable in-country capacity

Sustainable in-country capacity is needed for diagnostics to respond in the intermediate and long-term infectious diseases threats. Higher-cost commercial kits are unlikely to solve this issue at a national level in many resource-constrained countries. Therefore, key reagents, protocols, and quality control standards (e.g., proficiency panels) must be made available to national reference laboratories and other such public-sector entities to ensure wide and sustainable adoption.

Conclusions

Promising technologies for detection of ZIKV and DENV infections are currently in the pipeline. These technologies have the potential to address many of the current challenges of epidemic flaviviral diseases. The rate-limiting bottleneck is early access to calibration controls and screening panels as well as access to well-characterized samples for development, validation and comparison of the performance of different assays. Proficiency testing for both serological and molecular diagnostics should be developed for all endemic regions, paired with capacity building. We suggest that an international reference laboratory response for flaviviruses is needed, which would include networks of in-country laboratories and preparation of protocols for evaluation studies. This could be achieved through initiatives such as the Global Dengue and Aedes Transmitted Disease Consortium (GDAC), the European Virus Archive, the future EVD-LabNet by the European Centre for Disease Prevention and Control, or the Zika research consortia funded by the European Commission[49]. The knowledge obtained should be put into the public domain. Researchers and policy-makers alike need to ensure mechanisms for greater reagent availability and sharing of standard reagents such as reference materials, antigens, monoclonal antibodies, cell lines, control sera, and standardized protocols. While this workshop focused on challenges for arbovirus diagnostic development, the key outcomes highlighted here translate to all pathogens of epidemic potential.
Notes

Funding: The meeting and subsequent work was funded by the Gates Foundation, the European Union’s 2020 Research and Innovation Programme through the ZikaPLAN consortium (grant agreement 734584), the EPSRC IRC in Early-Warning Sensing Systems for Infectious Diseases (EP/K031953/1) and unrestricted grants from bioMérieux, Sanofi Pasteur, and Takeda. We received in-kind support from the Foundation for Innovative and New Diagnostics (FIND) and from WHO TDR and WHO Blueprint.

Acknowledgement: We would like to thank the Fondation Merieux for supporting the meeting, especially Richard Vaux, Benedicte Pansier and Severine Comignani for their assistance in all the logistics. Lastly, we would like to acknowledge Raman Preet’s assistance for all ZikaPLAN consortium related matters during this meeting.

Potential conflicts of interest The authors declare no conflicts of interest

References


19. Andrade DV, Harris E. Recent advances in understanding the adaptive immune response to Zika virus and the effect of previous flavivirus exposure. Virus Res 2017.


Table 1. Summary table of the challenges and drivers of the pathway to adoption

<table>
<thead>
<tr>
<th>Step*</th>
<th>Challenges</th>
<th>Drivers</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Market failure due to uncertainty and lack of demand of public health emergencies.</td>
<td>R&amp;D models for diagnostic preparedness - including product development; Product development partnerships (PDPs) such as CEPI.dx; Other innovative financing models.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Target Product Profiles (TPPs)</td>
<td>Risk and benefit models to set accuracy targets may help inform use of diagnostic tests when they do not meet the minimum or ideal characteristics set in the TPP.</td>
</tr>
<tr>
<td></td>
<td>- The performance characteristics that are set in the TPP are aspirational in nature</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Often deemed to be too stringent</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Lack of clinical samples and resources for clinical validations.</td>
<td>Development of international reference standard for assay comparability; Improved access to qualified field laboratory networks; Access to proficiency panels; Development of standardized protocols.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Regulatory approval that is region-specific, non-transparent, complex, slow and costly.</td>
<td>Establishment of regulatory networks, common strategies, information-sharing and early-partnerships.</td>
</tr>
<tr>
<td>E</td>
<td>Limited in-country capacity for wide adoption.</td>
<td>Mechanisms for appropriate transfer of technology in a more stream-lined fashion; Regulation of quality of local laboratories and “in-house” assays for national scale-up and sustainable implementation.</td>
</tr>
</tbody>
</table>

*Steps along the pathway to adoption that require optimization (Figure 4 A-E)
Isolation of first dengue virus

During World War II (WWII) in the Pacific, thousands of soldiers from Allied and Japanese forces succumbed to diseases such as dengue and malaria.

Both armies appointed commissions to study these diseases. Albert Sabin and Susumu Hotta headed the Allied and Japanese commissions, respectively. Almost simultaneously they have isolated the first dengue virus (DENV-1).

Development of first serological test

Sabin’s group developed the first serological test for dengue - the precursor of the hemagglutination-inhibition test that has been used for many years for dengue diagnosis.

Development of mosquito inoculation technique

Duane J Gubler and Leon Rosen developed the mosquito inoculation technique which, for the first time, provided a highly sensitive method for isolating dengue virus from human clinical material.

Development of IgM test

The IgM ELISA was developed, along with the use of serotype-specific monoclonal antibodies for identification of isolated viruses.

Development of PCR test

In the early 1990s, Robert Lanciotti, Duane J Gubler and Charles Calisher developed the first PCR assay for dengue - a nested RT-PCR still widely used today.

Figure 1. Historical timeline of dengue diagnostics 1940s-1990s. Dengue virus was first isolated in the early 1940s by the groups of Albert Sabin and Susumu Hotta. A number of viral isolation, serological and molecular methods have been developed since then. IF Immunofluorescence; ELISA Enzyme-Linked Immunosorbent Assay; IgM Immunoglobulin M; RT-PCR Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)
A) Assays to detect the pathogen:
- Viral Isolation
- Viral Nucleic Acid testing (NAT)
- Viral antigen detection

B) Assays to detect exposure to the pathogen:
- IgM/IgG detection by ELISA/RDT
- Plaque Reduction Neutralization Tests (PRNT)

C) Combination assays: Antigen + IgM/IgG detection

Figure 2. Schematic representation of the typical kinetics of flaviviral infections (adapted from Peeling et al 2010 with permission). As for most arboviral infections, viremia (red line) normally precedes the onset of clinical symptoms (0) and lasts for a few days after symptom onset. During the acute phase, flaviviral infections are best detected by viral isolation, NAT and antigen detection assays (e.g. DENV NS1) (A). During the course of infection, viral-specific IgM (blue line) and IgG (green line) antibodies are produced and can be used to detect exposure to the pathogen (B). Current infections can be diagnosed by the detection of IgM and IgG antibodies in paired acute and convalescent samples (detection of seroconversion or a >4-fold rise in IgG or total antibody titer). IgM antibodies can persist for several months, and IgG antibodies are known to persist for several years. Combination assays that detect both antigen and antibodies are applicable throughout the entire spectrum of disease (C).
A) Assays to detect the pathogen

**Viral isolation:**
- **Application**: Detection of active infection. Interpretation of positive result: confirmatory. Allows identification of viral species and DENV serotypes.
- **Advantages**: Most specific and conclusive diagnosis; only method that allows the detection and isolation of living virus.
- **Limitations**: Lab-based; requires acute sample; takes more than one week to complete; expensive; laborious and impractical in point-of-care and resource-limited settings.

**Nucleic acid testing (NAT):**
- **Application**: Detection of active infection. Interpretation of positive result: confirmatory. Allows identification of viral species and DENV serotypes.
- **Advantages**: Highly accurate if performed correctly; faster and less laborious than viral isolation; multiplex and near point-of-care testing possible.
- **Limitations**: Mainly lab-based; requires acute sample; complex; requires power supply; expensive; potential false positives.

**Antigen detection (DENV NS1):**
- **Application**: Detection of active infection - interpretation of positive result: confirmatory.
- **Advantages**: Diagnostic window of DENV NS1 up to day 8 post-onset of DENV infection with less sensitivity; ELISA (high throughput) or RDT; can be used at or near the point-of-care; easy to perform; less expensive.
- **Limitations**: Less accurate than viral isolation and NAT, requires acute sample, variable results according to serotype and immune status. No antigen detection assay available for ZIKV at present.

B) Assays to detect exposure to the pathogen

**Serology:**
- **Application**:
  - *IgM/IgG (paired serum specimens)*: Detection of active infection upon serconversion (negative IgM/IgG in an acute specimen followed by a positive IgM/IgG in a convalescent specimen) or a ≥4-fold rise in IgG or total antibody titer, interpretation of positive result: confirmatory.
  - *IgM (single serum specimen)*: Detection of recent past infection. Interpretation of positive result: probable. Useful for surveillance.
  - *IgG (single serum specimen)*: Detection of past infection. Classification of primary or secondary infections (IgG avidity, IgM/IgG ratio, titer).
  - *Plaque reduction neutralisation test (PRNT)*: Detection of past infection. Identification of viral species and infecting DENV serotypes in primary infection and some secondary infections. Useful for research and vaccine studies.
- **Advantages**:
  - *IgM/IgG (single/paired serum specimens)*: Can be used as ELISA (high throughput) or as RDT at or near the point-of-care; easy to perform; affordable.
  - *PRNT*: Higher specificity than IgM/IgG assays.
  - *IgG (single/paired serum specimens)*: Possible identification of infecting viral species and DENV serotypes during the convalescent phase; indicates the level of protection against an infecting virus.
- **Limitations**:
  - *IgM/IgG (single/paired serum specimens)*: Limited specificity due to high cross-reactivity among Flaviviruses.
  - *PRNT*: Limited sensitivity during acute phase of infection; Testing single serum specimens is not confirmatory of current infection; Confirmatory results can only be obtained after a second visit (convalescent samples are difficult to obtain); Variation among laboratories.
  - *IgG (single/paired serum specimens)*: Time- and labor-intensive with variation among laboratories; Not always able to resolve cross-reactivity (particularly in secondary infections).

C) Combination assays

**Antigen (DENV NS1) & IgM/IgG combination assays:**
- **Application**: Detection of active infection. Interpretation of positive result: confirmatory.
- **Advantages**: Entire temporal spectrum following infection (applicable in acute and convalescent samples); RDT; can be used at or near the point-of-care; easy to perform; less expensive; can use whole blood.
- **Limitations**: Less accurate than viral isolation and NAT during acute phase. Variation among laboratories. No assays available for ZIKV at present.

Figure 3. Applications, advantages and limitations of the main diagnostic tests in use for the detection of flaviviral infections.

Assays to detect the pathogen directly (A): viral isolation; nucleic acid testing (NAT) and antigen detection assays; Assays to detect exposure to infection (B), Combination assays to detect both the pathogen and exposure to infection (C). *Please note that RNA copy number is not an accurate measure of infectious virus (viral RNA can persist for longer periods than infectious virus).*

---

21
Figure 4. Diagnostic landscape to detect ZIKV and DENV infections and the pathway to adoption. The Zika Public Health Emergency (PHE) triggered significant efforts towards the development of new diagnostic assays. Commercial kits and ‘in-house’ laboratory developed tests (LDTs) are available to detect ZIKV and DENV infections. Multiplex assays that simultaneously detect ZIKV and other arboviral infections (DENV, CHIKV and others) are also available. Target Product Profiles (TPP) are used to define the desired technical and operational characteristics of a test. Quality-assured clinical laboratories can develop, validate and then implement their ‘in-house’ LDTs. Commercial kits require clinical validation, scale production, distribution, and regulatory approval in order to be adopted into wide-use. Two DENV commercial kits (one NAT and one IgM capture ELISA) have been cleared by the FDA. Emergency use authorization mechanisms by the FDA (EUA) and the WHO (EUAL) were put into place to accelerate adoption of ZIKV commercial kits in response to the Zika PHE. In bold are the type of assays that are currently in the pipeline (most assays are stuck at the evaluation stage due to the lack of access to well-characterized clinical specimens); *assays that require laboratory infrastructure; **No RDT assay has been FDA cleared nor received EUA/EUAL to date; A-E steps identified along the pathway to adoption that need optimization;