# Flavivirus Diagnostics Landscape – Focusing on Dengue and Zika

**Maurine M. Murtagh**

**Workshop on New and Innovative Approaches to Laboratory Diagnosis of Zika, Dengue and Other Arboviruses**

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# Background

The definitive diagnosis of dengue and Zika viral infections is important for:

- clinical management of patients
- surveillance
- outbreak investigations

allowing for early interventions, to monitor or treat patients and to prevent or control epidemics.

But, they share common, non-specific symptoms and geographic vector distribution – making definitive diagnosis challenging.

Aedes aegypti (left) and Aedes albopictus (right) mosquitoes

# Clinical Manifestations of Dengue and Zika Virus Infections

Because of their non-specific symptoms, DENV and ZIKV infections are often confused.

Differences in symptoms between/among these and other flaviviruses are subtle.

Clinical manifestations and relevant epidemiologic exposure alone are not reliable indicators of either of these infections.

Yet, it is important to distinguish DENV and ZIKV infections:

- given the need to monitor patients for the onset of severe illness in the case of DENV
- need to inform pregnant women of the risk of transmission of ZIKV to the fetus and to recommend further testing (e.g., amniocentesis, fetal ultrasound).

# Clinical Manifestations of Dengue and Zika Virus Infections

| | DENV Infection | ZIKV Infection |
|---|---|---|
| Signs and symptoms | Maculopapular rash, myalgias and arthralgias, conjunctivitis; ZIKV often not symptomatic | |
| Temperature | >40° C, plus 2 of the following: severe headache, retro-orbital pain, myalgias and arthralgias, nausea and vomitng | Temp: 37.5°- 38.5°C, plus any 2 of symptoms above. May also see: joint swelling, headache, retro-orbital pain |
| Incubation period | ~4 – 7 days | Unknown, but may be similar to DENV |
| Duration of symptoms | Usually 2 – 7 days; may lead to severe DENV infection. | Usually 2 – 7 days; self-limiting |
| Clinical management | No specific treatment; close assessment of hydration, bleeding status, etc. | No specific treatment; management of symptoms |
| Long-term effects and sequelae | Documented for up to 2 years following illness. Link to GB and other neuro syndromes. | Link to microcephaly; possible link to GB, encephalitis, other neuro syndromes. |

# Diagnostic Methods for DENV and ZIKV Infections

Diagnostic methods for DENV and ZIKV infections:

- Detect the virus directly (virus isolation)
- Detect the components of the virus (RNA or antigen)
- Detect specific antibodies, generally IgM and IgG

Assay selection depends both on the timing of sample collection and the purpose of testing.

In the acute phase of infection (~first 7 days), assays that detect the virus directly, viral RNA or viral antigen, perform best.

In the convalescent phase of the infection, serological assays that detect antibodies perform best.

# Detecting Acute Infection – DENV and ZIKV

The most commonly used methods to diagnose DENV and ZIKV infections are to detect viral RNA in various specimens, including plasma or sera, by reverse transcriptase (RT) polymerase chain reaction (PCR), usually real-time RT-PCR.

Culture-based methods (viral isolation) to isolate each of these infections is possible, but the methods are laborious and impractical, especially in resource-limited settings.

In the case of DENV infection antigen (NS1), detection methods are also available and work best during the acute phase of infection; similar methods generally are not yet available for ZIKV infection.

# Detecting Non-Acute Infection – DENV and ZIKV

Antibody testing, namely IgM, IgG, and in the case of DENV infection, IgA, and neutralizing antibody testing, such as plaque-reduction tests (PRNT) are used.

ELISAs are the most frequently used tests for DENV; for ZIKV infection, both ELISAs and immunofluorescence assays (IFAs) that detect IgM are utilized.

The primary drawbacks to antibody testing are:

- not sensitive enough to confirm either DENV or ZIKV infection during the acute phase
- can be unreliable because of cross-reactivity between and among viruses (e.g., cross reactivity between DENV and ZIKV among individuals who have been previously infected, or vaccinated against, a related flavivirus).

# Diagnosing DENV and ZIKV Infections at the Point of Care

Most diagnostic methods for detecting DENV and ZIKV infections are laboratory-based.

Affordable diagnostics that can be used at or near the point of patient care (POC) are needed.

Tests that meet the ASSURED criteria would be ideal:

**A = Affordable**
**S = Sensitive**
**S = Specific**
**U = User-friendly (simple to perform in a few steps with minimal training)**
**R = Robust and rapid (results available in less than 30 minutes)**
**E = Equipment-free**
**D = Deliverable to those who need the test**

# Diagnosing DENV Infection

DENV infection has certain features that make it especially difficult to diagnose, including the need to:

- Distinguish between multiple serotypes;
- Distinguish between primary and secondary infection, due to increased risk of severe DENV infection in the case of secondary infection; and
- Differentiate between DENV infection, where prevalent, and other infections, including ZIKV, malaria, pneumonia or other infections (influenza, leptospirosis, measles, Japanese encephalitis, Yellow Fever virus, and chikungunya).

# Detecting Acute DENV Infection – RNA-based Testing

Most commonly used methods of detecting acute DENV infection today are NAATs, most of which use real-time RT-PCR (rRT-PCR).

Compared to RT-PCR, rRT-PCR testing:

- limits the need for post-amplification manipulations
- lowers the risk of contamination
- permits serotyping of DENV.

Still, most of these are “in-house” laboratory procedures, many of which have not been commercialized, nor have most undergone stringent QA.

Several laboratory-based RT-PCR DENV assays have been developed and commercialized. They are generally sensitive and specific, but cannot be performed at POC.

# Detecting Acute DENV Infection – Commercial RT-PCR Tests

In addition to an FDA-approved RT-PCR assay from the CDC, there are at least half a dozen other commercialized, NAAT-based assays.

Najioullah et al evaluated three of these:

| **Serotype** | **N** | **Geno-sen's (n (% [95% CI])** | **Realstar (n (% [95% CI])** | **Simplexa (n (% [95% CI])** |
|---|---|---|---|---|
| DENV-1 | 46 | 42 (91.3 [83.2-99.4]) | 36 (78.3 [66.4-90.2]) | **44 (95.7 [89.7-100])** |
| DENV-2 | 37 | 33 (89.2 [79.2-99.2]) | 32 (86.5 [75.5-97.5]) | **34 (91.9 [83.1-100])** |
| DENV-3 | 33 | 30 (90.9 [81.1-100]) | 30 (90.9 [81.1-100]) | **30 (90.9 [81.1-100])** |
| DENV-4 | 46 | 33 (71.7 [58.7-84.8]) | 37 (80.4 [68.9-91.9]) | **43 (93.5 [86.3-100])** |
| Total | 162 | 138 (85.2 [79.7-90.7]) | 135 (83.3 [77.6-89.1]) | **151 (93.2 [89.3 – 97.1])** |

Simplexa performed well and was suggested for further evaluation.

# Detecting Acute DENV Infection – Other Molecular Tests

Several molecular technologies have been adapted for DENV detection: NASBA-, LAMP-, TMA- and RT-RPA-based methods.

- Neither NASBA- or TMA-based tests have been commercialized yet for DENV infection;
- LAMP- and RT-RPA-based testing have been developed, but are not in widespread use.

These methods are simpler, more rapid and/or provide additional sensitivity compared to conventional PCR.

Hope for assays is that these technologies could be used at/near POC.

The technologies are promising and more appropriate for POC, but require further development and validation.

# Detecting Acute DENV Infection – Antigen Tests

Acute phase detection of DENV infection is also possible by detecting viral antigens in the bloodstream; in particular, the non-structural glycoprotein NS1, which has been shown to be present in high concentrations in the sera of DENV-infected individuals.

Many tests have been developed to diagnose DENV infection by detecting NS1, including both commercial ELISAs and RDTs. The RDTs, in particular, provide tests that are easier to use and more affordable than virus isolation and current commercial molecular assays.

A good number of performance evaluations of antigen-based DENV tests have been conducted.

Results vary by phase of infection (acute vs convalescent), by primary vs secondary infection and by type of test (ELISA or RDT).

# Performance of Antigen Tests – DENV Infection

| Tests | | Acute | | | Convalescent | | |
|---|---|---|---|---|---|---|---|
| | | **Positive** | **Total n = 107** | **Sensitivity[a] (95%CI)** | **Positive** | **Total n - 85** | **Sensitivity[b] (95%CI)** |
| **ELISA** | Platelia™ Dengue NS1 Ag | 64 | 106[c] | 60% (51-70) | 24 | 83[c] | 29% (19-39) |
| | Panbio® Dengue Early ELISA | 78 | 104[c] | 75% (67-83) | 16 | 84[c] | 19% (11-27) |
| | SD Dengue NS1 ELISA | 74 | 105[c] | 70% (62-79) | 26 | 85[c] | 31% (21-40) |
| **RDT** | Dengue NS1 Ag Strip | 104 | n=214 199[c] | 52% (45-59) | 32 | n=170 170[c] | 19% (13-25) |
| | *OnSite* Dengue Ag Rapid Test | 50 | 125[c] | 40% (31-49) | 33 | 170 | 19% (13-25) |
| | Dengue Early Rapid Test | 119 | 197[c] | 60% (54-67) | 21 | 170 | 12% (7-17) |
| | SD Bioline Dengue Duo | 115 | 195[c] | 59% (52-66) | 100 | 170 | 59% (51-66) |
| **Tests** | | **Primary** | | | **Secondary** | | |
| | | **Positive** | **Total n = 45** | **Sensitivity[a] (95%CI)** | **Positive** | **Total n - 147** | **Sensitivity[b] (95%CI)** |
| **ELISA** | Platelia™ Dengue NS1 Ag | 26 | 43[c] | 60% (46-75) | 62 | 147 | 42% (34-50) |
| | Panbio® Dengue Early ELISA | 28 | 43[c] | 65% (51-79) | 66 | 146[c] | 45% (37-53) |
| | SD Dengue NS1 ELISA | 33 | 44[c] | 75% (62-88) | 67 | 147 | 46% (38-54) |
| **RDT** | Dengue NS1 Ag Strip | 46 | n=90 78[c] | 59% (48-70) | 90 | n=294 293[c] | 31% (25-36) |
| | *OnSite* Dengue Ag Rapid Test | 37 | 78[c] | 47% (36-59) | 46 | 218[c] | 21% (16-27) |
| | Dengue Early Rapid Test | 30 | 78[c] | 38% (54-67) | 110 | 291[c] | 38% (32-43) |
| | SD Bioline Dengue Duo | 55 | 78[c] | 71% (60-81) | 160 | 289[c] | 55% (50-61) |

**Sensitivity of dengue NS1 antigen tests in acute and convalescent specimens (top table) and in primary and secondary DENV infections (table immediately above). Reproduced from Hunsperger et al . [a]Compared to RT-PCR DENV positive samples;[b]Compared to IgM seroconversion;[c]Number of samples tested different than total number due to either duplicates for RDTs, invalid test or equivocal result.**

# Conclusions on Antigen Test Performance

**NS1 ELISAs**: significant differences in performance based on acute/convalescent and primary/secondary infections:

- Acute phase: sensitivities ranged from 60 – 75%; Panbio® best.
- Post acute phase: sensitivities ranged from 19 – 31%; SD Dengue best.
- Primary infection: sensitivities ranged from 60 – 75%; SD Dengue best.
- Secondary infection: sensitivities ranged from 42 – 46%.
- Specificities (using DENV negative and challenge specimen panels) ranged from 71 – 80%.

**NS1 RDTs**:

- Acute phase: sensitivities ranged from 40-60%, Panbio® and SD best.
- Post acute phase: sensitivities ranged from 12 – 59%, with SD performing best, Panbio® worst.
- Primary infection: sensitivities ranged from 38 – 59%.
- Secondary infection: sensitivities ranged from 21-55%.
- Specificities ranged from 76 – 80%.

# General Conclusions on Antigen-only DENV Tests

NS1 ELISA assays typically perform better than NS1 RDTs. Trade-offs need to be considered.

All tests (ELISAs/RDTs) generally perform better in the acute phase of the infection (days 1 to 5 of fever onset); NS1 detection rates decrease as the IgM levels increase over days.

Tests can differentiate between primary and secondary infection, but assay sensitivities in secondary infection not particularly high.

Current commercial NS1 assays cannot differentiate between and among serotypes.

Performance and utility of NS1 assays require additional evaluation, and a standardized approach should be used.

# Detecting the Acquired Immune Response to DENV

Serological tests that detect dengue IgM and virus-specific IgG and IgA are the most frequently used tests in many endemic countries because they are easy to use compared to culture or NAATs and are relatively affordable.

Assays are best used during the convalescent period of the infection.

Although there are HI and PRNT assays for detection of DENV infection, they are labor intensive and difficult to use.

Will focus here on IgM, IgG and IgA ELISAs and RDTs.

# Serological Tests to Detect DENV Infection

The IgM antibody-capture ELISA (MAC-ELISA) is the most widely used method to detect DENV infection. But, it lacks the sensitivity and specificity to diagnose acute infection and is time consuming.

In addition to numerous home-brew MAC-ELISAs, there are more than 50 commercial kits. RDTs for detection of IgM are also available.

Numerous evaluations on both MAC-ELISA assays and IgM RDTs were done from the late 1990's through the mid-2000's.

Sensitivity and specificity have been shown to be highly variable: sensitivities and specificities ranging from 61.5% to 99.0% and 84.4% to 98.0%, respectively.

# Performance of DENV Serological Tests - RDTs

| Assay | Study | Year | Location | Reference | Antibody Target | Sensitivity (95%CI) | Specificity (95%CI) |
|---|---|---|---|---|---|---|---|
| SD Bioline Dengue Duo (2nd Generation) | Wang and Sekaran (*88*) | 2010 | Malaysia | Virus isolation, RT-PCR, paired MAC-ELISA | IgM | 53.5% | 100% |
| | Blacksell et al (*90*) | 2011 | Sri Lanka | AFRIMS* MAC- and GAC-ELISA paired samples | IgM | 79.2% (70.5-87.2) | 89.4% (83.5-93.7) |
| Panbio Dengue Duo Cassette | Blacksell et al (*117*) | 2006 | Thailand | AFRIMS MAC- and GAC-ELLISA paired samples | IgM | 65.3% (59.9-70.5) | 97.6% (93.9-99.3) |
| | Nga et al (*118*) | 2007 | Vietnam | Focus IgM/IgG ELISA | IgM<br>IgG | 67.3% (57.8-75.6)<br>66.4% (58.4-75.6) | 91.7% (84.4-95.7)<br>94.4% (84.9-98.1) |
| | Moorthy et al (*119*) | 2009 | South India | Panbio MAC- and GAC-ELISA | IgM<br>IgG | 81.8%<br>87.5% | 75.0%<br>66.6% |
| | Blacksell et al (*90*) | 2011 | Sri Lanka | AFRIMS MAC- and GAC-ELISA paired samples | IgM | 70.7% (60.7-79.4) | 80.0 (73.0-85.9) |
| Merlin IgM | Blacksell et al (*90*) | 2011 | Sri Lanka | AFRIMS MAC- and GAC-ELISA paired samples | IgM | 72.7% (62.9-81.2) | 73.8% (66.2-80.4) |
| Biosynex IgM | Blacksell et al (*90*) | 2011 | Sri Lanka | AFRIMS MAC- and GAC-ELISA paired samples | IgM | 79.8% (70.5-87.2) | 46.3% (38.3-54.3) |
| MP Diagnostics ASSURE | Tan et al (*111*) | 2011 | Singapore | NS1 Ag and MAC-ELISAs | IgA | 86.7% | 86.1% |

**Summary of selected recent assessments of dengue IgM, IgA and IgG antibody RDTs. Adapted from Blacksell et al (*86*).**

***Paired samples provided by the Armed Forces Research Institute of Medical Sciences (AFRIMS).**

# Performance of DENV Serological Tests

Sensitivities across all RDTs from all specimens (mix of acute patients and convalescent patients) ranged from 53.5 – 99.4%. Specificities across all tests ranged from 46.3% to 100%.

In a 2014 study by Hunsperger et al, performance of one IgM-based ELISA and four IgM-based RDTs were compared.

- Overall sensitivity of the ELISA was 96% and specificity was 84%.
- False positive reactions against other arboviruses, including Japanese Encephalitis, West Nile virus and CHIKV were observed.
- Performance mixed for RDTs: broad range of overall sensitivities (52 - 95%) and specificities (86% to 92%).

# General Conclusions on DENV Serological Tests

Across all studies, performance of MAC-ELISAs is better than the performance of IgM RDTs, although the heterogeneity in evaluation methodologies make comparisons among studies difficult.

Significant differences in sensitivity and specificity among the commercial assays, particularly RDTs, has been called “very disheartening.”

IgM-based RDTs for DENV are not sensitive enough to diagnose acute DENV infection; paired sera must be used.

Neither MAC-ELISAs nor IgM-based RDTs can determine DENV serotype.  Both are subject to cross reactivity.

# Detecting DENV Infection – Combined NS1 Antigen and IgM/IgG Assays

In order to enhance performance of DENV tests, combined NS1 antigen and IgM/IgG assays have been developed. Most of these are ELISAs, but one test is an RDT.

A good number of studies have been performed on both ELISA and RDT combination assays.


**SD Dengue Duo RDT**

# Performance of Combined DENV NS1 Antigen and IgM/IgG ELISA Assays

| | Sensitivity (95%CI) for: | | | |
|---|---|---|---|---|
| **Assay(s)** | **Admission samples (n=239)** | **Discharge samples (n=239)** | **All samples (n=626)** | **% specificity (95%CI)** |
| *Dengue NS1 detection ELISAs* | | | | |
| Panbio® Dengue Early ELISA | 44.8% (38-51) | ND* | 44.8% (38-51) | 93.2% (88-97) |
| SD Dengue NS1 Ag ELISA | 55.2% (49-62) | ND | 55.2% (49-62) | 98.6% (95-100) |
| Platelia™ NS1 Antigen ELISA | 56.5% (50-63) | ND | 56.5% (50-63) | 100% (98-100) |
| | | | | |
| *Dengue IgM detection ELISAs* | | | | |
| Panbio® Dengue IgM Capture ELISA | 83.2% (78-87) | 93.7% (90-96) | 88.6% (86-91) | 87.8% (82-93) |
| SD Dengue IgM Capture ELISA | 74.4% (69-80) | 95.0% (91-97) | 84.9% (81-88) | 97.3% (93-99) |
| | | | | |
| *Dengue IgG detection ELISAs* | | | | |
| Panbio® Dengue IgG Capture ELISA | 39.8% (4-46) | 72.8% (67-78) | 56.4% (52-61) | 95.3% (91-98) |
| SD Dengue IgG Capture ELISA | 81.2% (76-86) | 96.2% (93-98) | 88.9% (86-92) | 63.5% (55-71) |
| | | | | |
| Combined dengue IgM antibody and NS1 detection ELISAs | | | | |
| Panbio® | 87.9% (83-92) | ND | 87.9% (83-92) | 84.5% (78-90) |
| SD | 87.4% (83-91) | ND | 87.4% (83-91) | 95.6% (91-99) |

***Not determined**

Based on an evaluation of seven commercial antigen and antibody ELISAs for detection of acute DENV infection, Blacksell et al concluded **there is value in combining antigen and antibody assays in ELISA format**.

# Performance of Combined DENV NS1 Antigen and IgM/IgG ELISA Assays (cont'd)

Blacksell's study highlights that:

- Sensitivities of NS1 antigen, IgM or IgG assays alone are insufficient to diagnose DENV infection;
- Combining NS1 antigen and IgM antibody testing "provides the ideal balance of high sensitivity and specificity;"
- Offers the possibility of acceptably high levels of accuracy across the entire temporal spectrum of DENV infection.

And:

- IgG assays alone have poor diagnostic value for diagnosis of acute DENV infection;
- Still need to be aware of the possibility of false positive results due to persistence of DENV IgM antibodies from a previous DENV infection; and
- While the assays were able to detect all four DENV serotypes, five of seven assays demonstrated significant differences in positivity for the different serotypes.

# Performance of Combined DENV NS1 Antigen and IgM/IgG RDT Assays

There is now one combination NS1/IgM/IgG-based assay from Alere, the SD Dengue Duo RDT; can also use combination of Panbio® assays.

Quite a good number of studies have been performed on these assays over the last few years.

| Type of antibodies or antigens | Test | Sensitivity (95%CI) | Specificity (95%CI) |
|---|---|---|---|
| IgM antibodies | SD | 79.2% (70.5-87.2) | 89.4% (83.5-93.7) |
| | Panbio® Dengue Duo Cassette (IgM/IgG) | 70.7% (60.7-79.4) | 80.0% (73.0-85.9) |
| | | | |
| NS1 antigen | SD NS1 Ag | 48.5% (38.5-58.7) | 99.4% (96.6-100) |
| | Panbio® Dengue Early Rapid | 58.6% (48.2-68.4) | 92.5% (87.3-96.1) |
| | | | |
| IgM antibodies and NS1 antigen | SD Dengue Duo RDT (NS1 with IgM/IgG) | 92.9% (83.9-97.1) | 88.8% (82.8-93.2) |
| | Panbio® combination | 89.9% (82.2-95.0) | 75.0% (67.6-81.5) |

Blacksell et al (data above) found sensitivity of the combined assay from SD to be 92.9% and from Panbio® to be 89.9%.

# Performance of Combined DENV NS1 Antigen and IgM/IgG RDT Assays (cont'd)

Pal et al found that the SD Dengue Duo had an overall sensitivity of 87.3% and specificity of 86.8% during the first 14 days post-symptom onset (p.s.o.); the Panbio® Dengue Duo had an overall sensitivity of 92.1% and specificity of 62.2% during days 4 – 14 p.s.o.

Additional studies have shown mixed results, with some studies finding that the SD Dengue Duo has a significant weakness at classifying secondary infections (Piedrahita, Krishnananthasivam); Pal's study demonstrates otherwise.

General conclusion: Combination assays (both ELISA and RDT) is that *using them can increase the sensitivity of acute DENV diagnosis and can enhance classification of primary and secondary infection*.

But, beware significant heterogeneity among studies.

# Research Needs on Combined DENV NS1 Antigen and IgM/IgG Assays (ELISAs and RDTs)

Further research is needed:

- In non-endemic settings, to examine potential differences between pediatric and adult DENV-positive patients and their effects on the tests;
- To determine the best antigens for detection;
- To more fully assess performance across all four DENV serotypes;
- To address the issue or cross reactivity/false-positive tests; and
- To address the loss of specificity that sometimes occurs in combination testing.

# Summary of Types of DENV Tests

| Test | Days post-onset of sample collection | Interpretation of positive result | Serotype identification; Primary/Secondary Infection | Explanation | Comment |
|---|---|---|---|---|---|
| **Virus Isolation** | ≤5 days | Confirmatory | Yes; No | Requires acute sample (0-5 days) | Lab-based; complex; takes more than 1 week |
| **RNA detection** | ≤5 days | Confirmatory | Yes; No | Requires acute sample (0-5 days) | Lab-based; complex; potential false positives |
| **NS1 detection** | ≤5 days | Confirmatory | No; Yes | Best results on acute specimen, but can detect up to day 8 post onset of infection with less sensitivity | Lab or RDT; easy to perform; less expensive than virus isolation or RNA detection; less sensitive than RNA detection |
| **IgM (paired specimens, acute and convalescent)** | ≤5 days for acute specimen, > 5 days for convalescent, ideally at least 2 weeks apart | Confirmatory | No; Yes | Negative IgM in an acute specimen followed by a positive IgM in a convalescent specimen | ELISA or RDT; easy to perform; affordable; takes 72 hours for results |
| **IgG (paired specimens, acute and convalescent)** | ≤5 days for acute specimen, > 5 days for convalescent, ideally at least 2 weeks apart | Confirmatory | No; Yes | Negative IgG in an acute specimen followed by a positive IgG in a convalescent specimen or a 4-fold increase in titer between acute and convalescent specimen and confirmed by PRNT | ELISA or RDT; easy to perform; affordable |
| **IgM (single serum specimen)** | >5 days | Probable | No; Yes | Positive IgM in convalescent specimen | IgM levels can be low in secondary infection; Useful for surveillance |
| **NS1 and IgM/IgG assays used in combination** | Entire temporal spectrum of dengue | Confirmatory | No; [Yes] | Further research needed on the ability to distinguish primary/secondary dengue infection | RDT; easy to perform; affordable; can use whole blood |

***Courtesy or Rosanna Peeling, LSHTM***

# Diagnosing ZIKV Infection

The complexities:

ZIKV infection is difficult to distinguish from DENV and certain other infections. This complicates its definitive diagnosis.

There is no “gold standard” IVD for the confirmation of ZIKV infection.

Serologic assays are unreliable because antibodies among flaviviruses are often cross reactive.

There are no commercially available antigen-based assays.

# Detecting Acute ZIKV Infection – RNA Testing

RT-PCR, specifically rRT-PCR assays, are the laboratory diagnostics of choice for detection of acute ZIKV infection. A number of these assays have been developed and evaluated; most are in-house/home brew assays available for research use only. These are summarized below:

| Reference or Source | Year | RT-PCR type | Target* | ZIKV lineage analytical | ZIKV lineage field | #Human patients | Sample types |
|---|---|---|---|---|---|---|---|
| **Lanciotti et al** | 2008 | One step, real time; hydrolysis probe | prM | Asian, African | Asian | >200 (combined set) | Serum, urine, amniotic fluid |
| **Lanciotti et al** | 2008 | One step real time; hydrolysis probe | E | Asian, African | Asian | >200 (combined set) | Serum, urine, amniotic fluid |
| **Faye et al )** | 2008 | One step, conventional | E | African | Asian | >15 | Serum |
| **Balm et al.** | 2012 | One step, conventional | NS5 | African | Asian | 88 (none tested positive for ZIKV) | Plasma |
| **Faye et al** | 2013 | One step, real time, LNA probe | NS5 | Asian, African | African | 0 (only validated in monkeys) | Serum |
| **Tappe** | 2014 | One step, real time; hydrolysis probe | NS3 | Asian | Asian | 5 | Serum |
| **Pyke et al** | 2014 | One step, real time | E | Asian | Asian | 1 | Serum |
| **Pyke et al** | 2014 | One step, real time | NS1 | Asian | Asian | 1 | Serum |

***prM, precursor membrane; E, envelope; NS5, nonstructural protein 5; NS3, nonstructural protein 3; NS1, non-structural protein 1. Adapted from Charrel and Waggoner.**

# Detecting Acute ZIKV Infection – RNA Testing

Of the RT-PCR/rRT-PCR assays listed above, the most widely-evaluated assay is one developed by the US CDC – generally called the CDC ZIKV rRT-PCR assay.

The other methods described above have also been evaluated, but the literature is sparse.

In general, this is an issue with assays for ZIKV. There are very few performance evaluations of the assays.

# Detecting Acute ZIKV Infection – Commercial Tests

Relatively recently, molecular assays for ZIKV detection have become commercially available.

| Product | EUA | EUAL | CE-IVD | Review |
|---|---|---|---|---|
| RealStar® Zika Virus RT-PCR Kit 1.0 (altona Diagnostics GmbH, Germany) | ✓ | ✓ | ✓ | ✓ |
| *AccuPower*® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer, Republic of Korea) | | ✓ | ✓ | No |
| Zika Virus Real Time RT-PCR Kit (Liferiver/Shanghai ZJ Bio-Tech, China) | | Pipeline | | No |
| Abbott RealTi*m*e Zika (Abbott Molecular Inc., U.S.) | ✓ | | | No |
| Zika Virus RNA Qualitative Real-Time RT-PCR (Focus Diagnostics, Inc., a subsidiary of Quest Diagnostics, U.S.) | ✓ | | | No |
| Aptima® Zika Virus Assay (Hologic, Inc., U.S.) | ✓ | | | No |
| Zika Virus Real-time RT-PCR Test (Viracor-IBT Laboratories, Inc., U.S.) | ✓ | | | No |
| VERSANT® Zika RNA 1.0 Assay (kPCR) Kit (Siemens Healthcare Diagnostics Inc., U.S.), | ✓ | | | No |
| xMAP® MultiFLEX™ Zika RNA Assay (Luminex Corporation, U.S.) | ✓ | | | No |
| Sentosa® SA ZIKV RT-PCR Test (Vela Diagnostics U.S., Inc., U.S.) | ✓ | | ✓ | No |
| Zika Virus Detection by RT-PCR Test (ARUP Laboratories, U.S.) | ✓ | | | No |
| Gene-RADAR® Zika Virus Test (Nanobiosym Diagnostics, Inc., U.S.) | ✓ | | | No |
| Genesig® Easy Kit (Primerdesign™ Ltd., UK) | | | | No |
| Zika Virus – Single Check (Genekam Biotechnology AG, Germany) | | | ✓ | No |
| FTD Zika Virus (Fast Track Diagnostics, Luxembourg) | | | ✓ | No |

# Observations on Commercially-available Molecular Tests

All of the assays in the table are for use on laboratory-based instruments. They are not well suited for use in resource-limited settings, except at NRLs.

For the EUA-approved assays, testing is to be conducted only on specimens from individuals that meet the CDC ZIKV clinical and/or epidemiological criteria for testing (available at http://www.cdc.gov/zika/hc-providers/index.html) by laboratories in the U.S. that are certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) to perform high complexity tests, or by similarly-qualified laboratories outside of the U.S. Testing availability is very limited.

There is almost no independent performance data available on any of these assays. More evaluations studies are required.

# Detecting the Acquired Immune Response to ZIKV

Similar to DENV, there are serological tests that detect ZIKV IgM and virus-specific IgG.

Antibody assays for the diagnosis of ZIKV infection include ELISAs (e.g., one developed by the CDC) and immunofluorescence assays (IFAs). In addition, PRNT assays may be used.

Of these, IgM ELISAs are most frequently used. Commercial IgM antibody tests, including RDTs, for ZIKV are just becoming available.

Performance data on the assays is scarce.

# Commercially Available Antibody Assays for ZIKV

Commercially available serological tests for ZIKV include the following, some of which are RDTs:

| Product | EUA | EUAL | CE-IVD | Review |
|---|---|---|---|---|
| **Zika MAC-ELISA (CDC, U.S.)** | ✓ | | | No |
| **ZIKV Detect™ IgM Capture ELISA (InBios International, Inc. U.S.)** | ✓ | Pipeline | | No |
| **NovaLisa® Zika Virus IgM µ-capture ELISA (NovaTec Immunodiagnostica GmbH, Germany)** | | Pipeline | ✓ | No |
| **Anti-Zika Virus ELISA (IgM or IgG) (EUROIMMUN, Germany)** | | Pipeline | ✓ (RUO) | No |
| **STANDARD E Zika IgM ELISA (SD Biosensor Inc., Republic of Korea)** | | Pipeline | | No |
| **LIAISON® XL Zika Capture IgM Assay (DiaSorin Incorporated, U.S.)** | ✓ | | | No |
| **STANDARD Q Zika IgM/IgG Test (RDT) (SD Biosensor Inc., Republic of Korea)** | | Pipeline | | No |
| **DPP® Zika IgM/IgG Assay (Chembio Diagnostic Systems, Inc., U.S.)** | | Pipeline | ✓ | No |
| **TELL ME FAST Zika Virus IgG/IgM Antibody Rapid Test (Biocan, Canada)** | | | ✓ | No |

# Observations on Commercially Available Antibody Tests for ZIKV

There are only a few commercially available ZIKV antibody tests.

In the U.S., ZIKV antibody assays are available only through the CDC and other public health laboratories. Some of the tests are CE-IVD marked.

Given the association of ZIKV with GB and fetal microcephaly, improved access to quality-assured ZIKA-specific serological tests is required for clinical management.

All commercially available (and pipeline tests), whether laboratory-based or for POC, will require systematic clinical evaluation of test specificity, particularly in populations with high prevalence of other flaviviruses (particularly, DENV).

# Multiplex Diagnostic Assays for Simultaneous Detection and Differentiation of DENV and ZIKV

Several polyvalent assays to diagnose and differentiate DENV, ZIKV (and CHIKV) infections have been developed or are in development.

| Product | EUA | EUAL | CE-IVD | Review |
|---|---|---|---|---|
| **Molecular Assays** | | | | |
| **Trioplex Real-time Rt-PCR (Thermo Fisher Scientific, U.S.)** | ✓ | | | ✓ |
| ***AccuPower*® ZIKV (DENV, CHIKV) Multiplex Real-Time RT-PCR Kit (Bioneer, Republic of Korean)** | | ✓ | ✓ | No |
| **VIASURE Zika, Dengue & Chikungunya Real Time PCR Detection Kit (Certest Biotec, Spain)** | | | | No |
| **FTD Zika/Dengue/Chik assay (Fast-Track Diagnostics, Malta)** | | | ✓ | No |
| ***DiaPlexQ*™ ZCD Virus Detection Kit (SolGent Co., Ltd., Republic of Korea)** | | | | No |
| **Immunoassays** | | | | |
| **IIFT Arbovirus Fever Mosaic 2 (IgG or IgM) (EUROIMMUN AG, Germany)** | | Pipeline | ✓ | No |
| **recomLine Tropical Fever IgG; recomLineTropical Fever IgM (MIKROGEN Diagnostik, Germany)** | | | ✓ | No |
| **STANDARD Q Zika/Dengue Trio (SD Biosensor, Republic of Korea)** | | Pipeline | | No |
| **TELL ME FAST Dengue, Chikungunya & Zika Virus Combo test (Biocan Diagnostics Inc., Canada) (RDT)** | | | | No |

# Future Directions for Improving Access to DENV and ZIKV Testing – Diagnosis of Acute Infection

NAAT-based platforms for use at or near POC have the potential to be used for diagnosis of both acute DENV and acute ZIKV infection.

To date, only the Truelab™ Real Time micro PCR System (Molbio Diagnostics, India), pictured below, has a DENV assay on such a platform.

Other manufacturers have not developed DENV or ZIKV assays for these platforms and would need to be encouraged to do so.

# NAAT-based POC/near POC Platforms

Additional POC/near-POC molecular platforms for which such assays could be developed include:


**Alere™-i Platform**
**Alere**

**GeneXpert® Omni Platform**
**Cepheid**


**cobas® Liat System**
**Roche Molecular**

These are just a few of the new NAAT and iNAAT platforms either available or in development, any of which could be used to confirm acute DENV and/or ZIKV infection. But, none of these manufacturers has definite plans to develop such assays.

# Future Directions for Improving Access to DENV and ZIKV Testing – Detecting the Acquired Immune Response

There is a lot of activity in development of ZIKV and combination DENV/ZIKV assays, including antibody assays.

There are some 260 IVD companies and 14 pharma companies at present across Europe/US/Asia that are trying to develop diagnostic solutions for ZIKA in lateral flow, EIA, IFA, and chemiluminescence.

For example, bioLytical is developing the INSTI Zika (Arbovirus) Total Antibody test. It is a manual, visually read, 60-second POC assay for the qualitative detection of antibodies to ZIKV, DENV and CHIKV viruses in human EDTA whole blood, finger stick blood, serum or plasma.

Chembio is developing a lateral flow assay using its DPP® technology for the simultaneous detection of DENV, ZIKV and CHIKV infection.

# Next Generation Diagnostic Technologies

Diagnostic platforms/tests are also being developed using a variety of next generation technologies that may make it possible to further enhance diagnostic capabilities at POC.

- Examples include: techniques that permit microscale fabrication and processing methods using silicon and advances in plastics engineering.
- These can facilitate mass-produced, low cost, ultra-portable instrumentation with sophisticated sample and information processing capabilities that can be used effectively in diagnostics.

**PanNAT® Platform (Micronics)**

# Micro- and Nanoscale Detection Technologies

Offer a number of potential solutions. Some examples:

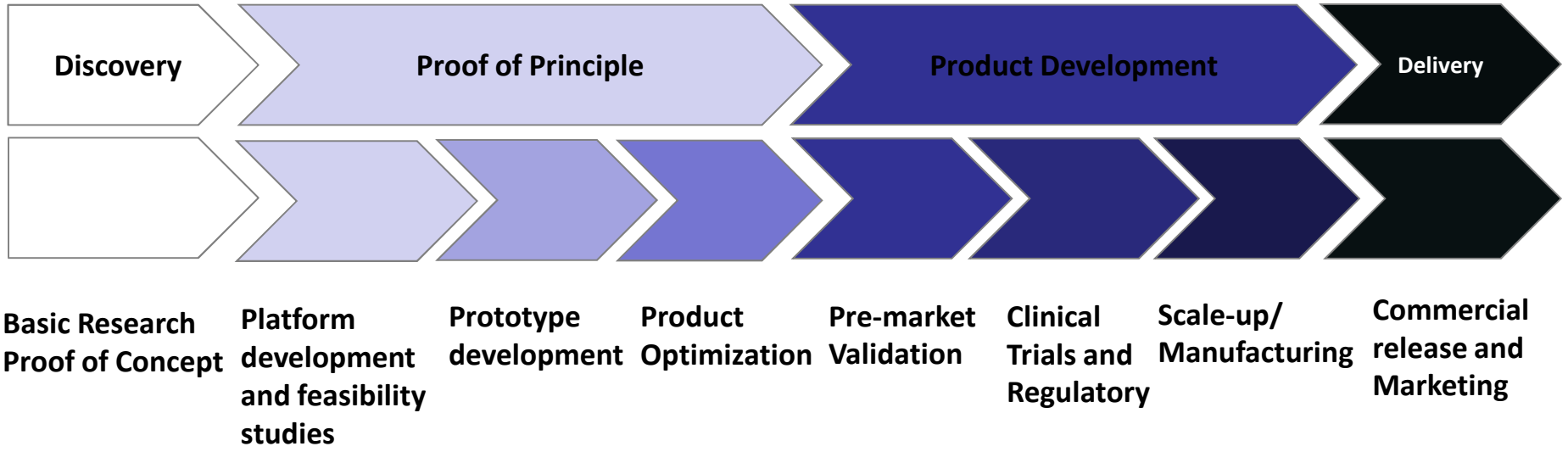
**PanNat®** (Micronics, Inc., U.S.) – microfluidic platform with fluorescent-based reader capable of processing individual, disposable, assay specific test cartridges that can perform multiplexed NAAT assays.

**IDAlert** (Aalto Bio Reagents, Ireland) – lab-on-a-chip technology, consisting of a handheld battery operated electronic reader and a sample assay chip card, that uses an electrochemical enzyme-linked immunoassay (EEIA) technology with an immune-electrode detector.

**BluSense Platform** (BluSense Diagnostics, Denmark) - a nano-technology platform that uses microfluidics and an opto-magnetic nanoparticle-based readout technology to detect viral RNA, antigen NS-1, and specific IgG/IgM antibodies.

# Observations on Micro- and Nanoscale Detection Technologies

Have potentially promising applications for diagnostics at POC, but to date, few have been commercialized – and this is a long, arduous road.




**And the process can easily take 5 - 7 years or more, even after prototype development, and can cost from $5 million to $50 million or more.**

# Conclusions

There is a need for quality-assured diagnostics for each of DENV and ZIKV infections.

Diagnostics for DENV infection have improved over the last decade and performance of combination NS1 and IgM/IgG testing is promising.

Despite recent activity, there are few commercially-available tests of any type for ZIKV and very poor access. Assays that are available have not been adequately and independently evaluated using clinical specimens. These studies are badly needed.

For both DENV and ZIKV, still need to continue to develop more:

- sensitive
- easy-to-use
- rapid and affordable tests
- that are, at the same time, serotype-specific and less cross-reactive.

# Thank you