

Diagnosis of Zika Virus Infections: Challenges and Opportunities

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Accurate diagnosis of Zika virus (ZIKV) infections has become a pressing need for the effective prevention and control of the epidemic. The findings that ZIKV infections are associated with birth defects and neurologic disease, and that the virus can be sexually transmitted, accentuate the need for accurate diagnostic testing for different applications new to the arbovirus field. Antibody response to related flaviviruses has long been known to be cross-reactive, and antibody detection of ZIKV is nonspecific in populations previously exposed to any of the four dengue viruses or West Nile virus, or vaccinated against yellow fever virus. Therefore, the diagnosis of ZIKV infections has increasingly depended on detection by nucleic acid tests. During the recent epidemic, tests authorized for emergency use have been utilized by public health laboratories and the commercial sector, but a more dependable and responsive diagnostic testing has yet to be developed.

Keywords. Zika; diagnosis; arbovirus; ZIKV.

THE NEED FOR ZIKA VIRUS DIAGNOSTIC TESTING

The particular needs for Zika virus (ZIKV) diagnosis have no precedent in arbovirology. The association of ZIKV infections with birth defects [1, 2] and neurologic disease [3, 4], and its potential spread through sexual transmission [5–8] have changed the paradigm of arbovirus diagnosis. Never before has diagnostic testing for an arboviral disease been required for disease surveillance, case confirmation, and screening of pregnant women for indication of current, recent, or past infection. ZIKV affects countries where the virus is transmitted as well as those receiving travelers returning from affected areas. Whereas testing of symptomatic individuals provides helpful information for health practitioners to identify cases and for public health agencies to alert communities and strengthen surveillance systems, the need for ZIKV detection is not limited to symptomatic infections. A high proportion of individuals infected with ZIKV are asymptomatic, and due to the risk of congenital abnormalities associated with ZIKV infection [9–14], prolonged viremia in pregnant women [15], and documented sexual transmission [6, 8, 16–19] testing algorithms must also account for large groups of asymptomatic individuals. Current guidelines from the Centers for Disease Control and Prevention (CDC) recommend the testing of pregnant women with possible exposure to the ZIKV during the first 12 weeks after travel and all persons with clinical illness and suspected exposure [20]. In areas with ongoing transmission, testing women throughout pregnancy is

recommended. CDC guidance also emphasizes the use of test results to counsel pregnant women, ascertain infection status of infants born to mothers with positive test results, and inform women and men who want to conceive [21–23]. The recommendation by public health authorities in each country of the groups who require testing may vary according to risks associated with infection, prevalence, number of suspected cases, numbers of pregnant women, and laboratory capacity. Many countries with ongoing ZIKV transmission have prioritized the testing of symptomatic pregnant women or symptomatic women of reproductive age [24], as testing of asymptomatic pregnant women is challenging due to limited laboratory capacity to enable retesting these patients multiple times throughout their pregnancies. In addition, Guillain-Barré syndrome (GBS) cases associated with ZIKV infections also demand diagnostic testing; studies show that testing during acute neurologic illness can be useful for diagnosing GBS as a sequela of ZIKV infection [25–27].

Thus tests are needed for the diagnosis of febrile illness, neuropathic disease, and for the screening of pregnant women and women of reproductive age, and of certain classes of abnormal fetuses and births. Test results may also help healthcare professionals guide or counsel men and women who could be either potential transmitters or recipients of ZIKV through sexual intercourse. This represents a diverse and ambitious testing algorithm that has challenged public health agencies in endemic and nonendemic countries.

DETECTION OF ZIKV RNA

Detection of ZIKV RNA in serum is feasible during the first few days of the acute, febrile phase of infection, and reverse transcription polymerase chain reaction (RT-PCR) has provided the means to determine the etiology of ZIKV outbreaks [28, 29]. It

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The Journal of Infectious Diseases® 2017;216(S10):S951–6

Published by Oxford University Press for the Infectious Diseases Society of America 2017. This work is written by (a) US Government employee(s) and is in the public domain in the US. DOI:10.1093/infdis/jix502

is possible to detect viral RNA in urine for an additional 2–3 weeks [29–32]. Testing urine requires special consideration in order to maximize detection, because preanalytical variables, such as temperature and length of storage, can significantly affect the stability of this sample. Storage of urine at 4°C for periods longer than 48 hours may affect ZIKV RNA detection by RT-PCR and freezing at –80°C results in significant loss of detectable ZIKV RNA in low positive samples [33]. In some instances, ZIKV RNA has been detected more frequently or for longer times after onset of illness in urine than in serum by RT-PCR. For example, among 80 travel-associated cases in New York, ZIKV RNA was detected in serum and urine specimens from 19 (24%) and 50 (63%) individuals, respectively [34]. Similarly, results obtained in paired serum and urine samples from 55 patients in Florida showed detection of ZIKV RNA in 31 (56%) serum and 52 (95%) urine samples [35]. However, a study in Puerto Rico of 150 patients with confirmed ZIKV infection showed that the median time until the loss of ZIKV RNA detection was 14 days after the start of symptoms for serum and 8 days for urine, respectively, with 95% of participants having undetectable viral loads by 54 and 39 days, respectively [36]. The cumulative evidence shows that the combined sensitivity of serum and urine RT-PCR during the first 14 days of illness is nearly 75% of serologically confirmed infections. This evidence, and a number of ongoing studies by CDC and others, substantiate the recommendation for nucleic acid tests (NAT) testing during the first 14 days after onset of symptoms or exposure in simultaneously collected serum and urine samples [37]. Serum should always be collected in order to attempt ZIKV RNA detection in symptomatic and asymptomatic individuals and it should always accompany urine or other specimens [20].

The presence of ZIKV in multiple sample types, the ease of *in silico* test design, and the availability of highly sensitive methods and equipment that meet regulatory standards for *in vitro* diagnostic devices, have motivated molecular test developers to put forward an unprecedented array of NATs for the diagnosis of ZIKV disease or the screening of blood supplies. In the last year, the US Food and Drug Administration (FDA) has authorized approximately 10 of these tests for emergency use [38, 39]. In initial evaluations, at least some of these tests, including the FDA-authorized Trioplex test developed by CDC, showed equivalence with a previously published primer and probe in-house test developed by CDC [28]. The Trioplex kit provided by CDC to US and international public health laboratories includes primers and probes for detection of ZIKV RNA as well as chikungunya virus (CHIKV) and dengue virus (DENV) RNA. Test compositions are mostly proprietary, but they are likely to differ in test conditions, chemistries, polymerase enzymes used, and equipment choices. Most of these tests are authorized for use with serum or urine samples, or both, and some were developed for plasma or whole blood. Implementation and marketing of the tests is also variable, with

some being commercialized as RT-PCR kits [40] and others becoming available for use with specific instrumentation or at specific laboratories [37, 38]. Some of these tests are available for blood screening as well as for diagnosis of cases or screening of pregnant women [41, 42]. Laboratory-developed tests for simultaneous DENV, CHIKV, and CHIKV RNA detection have also been published, and tests with primer and probe formulations that, although not standardized, allow users to more freely adapt tests to their requirements [43]. Differences in test design and sample input complicate test performance and sensitivity comparisons. In an effort to guide potential users, the instructions for use of these tests include results obtained on sample standards provided by the FDA. From this information, it appears that NATs have a limit of detection of between 1×10^2 and 2×10^4 genome copies (or nucleic acid detectable units) per milliliter of sample (urine or serum). The differences in sensitivity may be due to multiple reasons. Clearly, one of the variables that most directly correlate with test sensitivity is sample input (amount of sample or viral RNA extract that is actually tested). A direct comparison of the limits of detection of CDC's in-house test, the Trioplex test, and other FDA-authorized tests for high (1 mL) and low (0.2 mL) inputs showed that these tests have limits of detection of 1×10^2 and 2×10^3 genome copies/mL, respectively, indicating that sample volume is an important factor for test sensitivity [41]. At a 95% confidence level, the limits of detection of the most sensitive tests are approximately 10^2 – 10^3 genome copies/mL, whereas at a 50% confidence level some tests detect 10^1 – 10^2 copies/mL [41, 42]. Importantly, test accuracy near the limit of detection of FDA-authorized diagnostic devices needs to be kept near 95%–100% with the use of standardized conditions in order to ensure the veracity of test results and usefulness for patient care.

Testing blood or blood-derived samples (eg, serum or plasma) and urine generally provides a sensitive test combination for the diagnosis of ZIKV disease cases and for assessment of pregnant women's exposure to ZIKV. Because ZIKV infections have been associated with acute neurologic symptoms and GBS, including fatal encephalitis [44], testing of cerebrospinal fluid (CSF) by RT-PCR and enzyme-linked immunosorbent assay (ELISA) has been indicated in order to establish ZIKV as the cause of these clinical outcomes [25, 45]. Although the added value of testing CSF in addition to blood/serum and urine is limited, if the CSF samples are obtained as part of the medical ascertainment of neuropathic cases, the results may add to the rigor of clinical studies for the better understanding of ZIKV-associated neuropathic disease [44]. A ZIKV-positive result in CSF in the absence of positive results in serum and urine is a conclusive indication of infection [46].

The risk of sexual transmission of ZIKV highlights the need to better understand the dynamics of viral persistence in semen in men who live in or travel to areas of ZIKV transmission. Detection of ZIKV RNA in semen has been extensively

documented, with a median duration of approximately 30 days, significantly longer than in serum or urine [36], and possibly extending in some men to 80–180 days [47–50]. The long-lasting persistence of ZIKV RNA in semen, the reports of sexually transmitted ZIKV from male travelers to their female partners in nonendemic areas, and the presence of infected spermatozooids in fractionated semen evidence the risks of sexually transmitted ZIKV and highlight this concern for pregnant women [19, 51]. How long the virus is infectious in semen or other body fluids is still being explored; information on this question could help provide better diagnostics and prevention guidelines. Various forms of sexual transmission have been documented, and guidance for prevention of sexual transmission recommends testing for women and men with potential exposure to ZIKV due to unprotected intercourse [51]. Detection of ZIKV RNA in vaginal fluids and saliva has been reported in less than 10% of cases [35, 36, 52, 53] and there is some documented evidence of ZIKV RNA in breast milk [54]. The significance of these findings in transmissibility of ZIKV is not yet understood. None of the FDA-authorized tests are available yet for semen, saliva, breast milk, or vaginal fluids, though investigational studies are underway.

ZIKV is transmitted in countries where DENV and CHIKV are also endemic. Simultaneous detection of DENV, CHIKV, and ZIKV is feasible by real time RT-PCR assays without significant loss in sensitivity [37, 55–57]. The availability of these tests for public health laboratories is of considerable importance in regions with transmission of these 3 viruses, or in laboratories receiving samples from travelers to these regions. While a CDC DENV-1–4 RT-PCR test approved by the FDA in 2013 continues to be available to public health laboratories, and a variety of ZIKV NATs, including the CDC Trioplex, could potentially be of interest, no authorized CHIKV RT-PCR test is available other than the Trioplex. This test has been evaluated and validated for serum, urine, cerebrospinal fluid, amniotic fluid, and whole blood, and in high (1 mL) and low (0.2 mL) input volumes, and with several RNA extraction procedures, equipment, and automation protocols commonly available in public health laboratories. The deployment of Trioplex kits in the United States and internationally has provided public health laboratories with a tool to detect Zika cases while maintaining surveillance for the other 2 arboviral diseases without an increase in costs compared to running just 1 of the 3 tests. As the Zika epidemic subsides, the need for simultaneous detection is an opportunity for other test developers to advance in the direction of multiple-pathogen detection systems.

DETECTION OF ZIKA VIRUS ANTIBODIES

Whereas substantial progress has been made in ZIKV molecular test development, there is still a great demand for sensitive and specific immunodiagnostic tests that serve the needs of endemic areas. Immunodiagnostics play an important role in ZIKV diagnosis due to the high proportion of asymptomatic

infections and wide time window for detection. However, because of cross-reactivity with other flaviviruses, ZIKV serology is challenging. Even before the epidemic in the Americas, IgM and IgG ELISA assays for ZIKV diagnosis were known to have performance limitations [58–62]. Detection of IgM is indicative of recent exposure to ZIKV, which may be helpful in diagnosis of symptomatic or asymptomatic individuals who are RT-PCR negative. During the early course of infection, ZIKV RNA may not always be detected by RT-PCR either because the viremic period has passed or because viremia is not high enough to be detected. Individuals with suspected previous exposure to ZIKV can be tested for immunoglobulin M (IgM) antibodies in serum using IgM antibody capture ELISA (MAC-ELISA), authorized by the FDA for emergency use. Although the CDC Zika MAC-ELISA is highly sensitive, results may often be difficult to interpret. For symptomatic cases, IgM tests are most sensitive after the first 8 days of illness, and diagnostic guidelines recommend IgM tests on negative RT-PCR serum samples during the first 14 days of illness and in all serum samples 14 days after symptom initiation or suspected exposure to ZIKV [20]. In most IgM detection tests, a negative result indicates that there is no sign of recent infection, taking into consideration the period of illness and the sensitivity and specificity of the test. But a positive MAC-ELISA result is only an indication of a recent flavivirus infection (presumptive positive) [63, 64]. Therefore, a confirmatory plaque reduction neutralization test (PRNT) is required for a conclusive diagnosis of recent ZIKV exposure in areas where dengue or other flaviviruses have circulated [28, 59]. PRNT is a laborious test that measures neutralizing antibodies for viruses in infected cell monolayers. To establish if a serum sample contains specific anti-ZIKV antibodies, neutralization of ZIKV infection using titrated serum from patients must be shown to be considerably higher than DENV neutralization [59]. One study showed that a primary DENV infection does not induce high-level cross-neutralizing antibodies, and that even in secondary infections, cross-reactivity, though more common, may not be durable [65]. However, data from Puerto Rico indicated that PRNTs may not provide accurate confirmatory test results in populations with high pre-exposure to DENV [22, 59]. Microsphere-based antibody affinity tests and microneutralization tests using fluorescent detection methods are still investigational, but offer promising tools to detect specific antibodies for ZIKV and other arboviruses [66–69].

Three serologic tests have thus far been authorized for emergency use by the FDA. The CDC Zika MAC-ELISA test is highly sensitive when serum samples are collected during the appropriate time, usually after a few days of symptom initiation or exposure and for approximately 120 days. The reasons behind CDC's MAC-ELISA high sensitivity/low specificity are the overnight incubation of the serum with the antigen and the use of whole virus antigen (Vero-cell culture antigen or COS-1 recombinant antigen), which captures a wide variety of antibodies in the assay.

The InBios Zika Detect IgM Capture ELISA, authorized by the FDA, also makes use of COS-1 recombinant antigen developed by CDC and shows similar sensitivity [70]. A source of specificity in the InBios test comes from the inclusion of other flavivirus antigens in the test, allowing assessment of differential detection of ZIKV antibodies. Comparatively, the CDC-MAC ELISA and the InBios Zika Detect Antibody Capture tests have similar sensitivity, and the InBios test may detect fewer false-positive dengue infections than the CDC test [70, 71]. Anti-NS1 antibody detection methods apparently bring a higher level of specificity to immunodiagnosics for Zika [72], though possibly at a cost in sensitivity [70]. The Liaison XL Zika Capture IgM test has also been authorized for use recently and it detects binding of IgM antibodies to ZIKV NS1-coated microparticles, and subtracts signal generated by the binding of IgG antibodies. The combined use of NS1 antigen and the exclusion of IgG antibody signal result in the high specificity of IgM detection in this test, and more field work will help determine its usefulness. More recently, from antibody competition studies, specific NS1 epitopes have been identified, which have been used to generate more-specific antigens that discriminate Zika from dengue infections in a “blockade-of-binding” (BOB) ELISA. In a study, the developers of the BOB assay showed that primary and secondary dengue infections were not detected by the assay, whereas nearly 92% of PCR-confirmed ZIKV infections were detected [73].

As the field of immunodiagnosics for Zika continues to expand, more complete comparisons of serologic assays using characterized sera from confirmed ZIKV-specific or DENV-specific RT-PCR test result will be necessary in order to better understand differences in sensitivity and specificity, as well as the potential and limitations of these tests. An ideal IgM test would be one that allows specific detection of ZIKV antibodies at high sensitivity in order to distinguish infection from DENV and other flaviviruses in asymptomatic, symptomatic, and postsymptomatic individuals, particularly in pregnant women and women of reproductive age. A persistent limitation of the current ZIKV IgM tests is that equivalent dengue tests are not offered, therefore even when a ZIKV IgM specific test result can be obtained, a negative or indeterminate sample cannot yet be tested with an equivalent dengue test. In areas of coendemicity, simultaneous Zika and dengue IgM testing may be advantageous. A recent development shows the potential of using combined antigens in a multiplex microsphere immunoassay (MIA), which brings together the sensitivity of the viral envelope protein and the specificity of NS1 and NS5 proteins [74]. This platform, which can be modified and customized, may provide the basis for accurate, differential diagnosis of flaviviruses, a concept that could also be explored in ELISA formats.

Opportunities to develop immunodiagnostic tests for Zika do not end with differentiating flaviviral IgM antibody detection. In countries that recently experienced large ZIKV outbreaks, the diagnostic value of IgM detection may need reconsideration, as

IgM antibodies may last for several months and therefore indicate an infection well before pregnancy. Detection of IgG antibodies could play an important role in establishing the immune status of women of reproductive age. Presence of IgG antibodies would indicate previous exposure and therefore natural protection against infection, whereas a negative IgG test result would inform these women of their potential risks to acquire ZIKV infection. Although cross-reactive antibodies against flaviviruses can be common, studies with dengue have shown that infected individuals develop antibodies that target unique epitopes, and such virus-specific antibodies are linked to protection against DENV [75]. Building on these approaches may help researchers define and map specific antigenic protein epitopes to develop more specific IgM and IgG tests for the diagnosis of ZIKV infections.

PERSPECTIVE

The recent emergence of Zika virus in the Americas has brought an unprecedented amount of interest to the diagnosis of arboviruses. The declaration by the World Health Organization in 2016 of Zika virus as a public health emergency of international concern has encouraged test developers to develop molecular and serologic diagnostic tests and make them available to the large community of public health and commercial laboratories, as well as clinical research groups. The diagnosis of ZIKV, DENV, and other flaviviruses is challenging in areas where more than one is being transmitted. The specificities of dengue tests after the ZIKV epidemic need to be reassessed, just as much as the specificity of ZIKV tests in populations exposed to DENV-1–4, and more emphasis needs to be placed on the specific detection of IgM and IgG antibodies to determine recent infections and preimmunity in populations at risk of infection. The new landscape of arbovirus diagnosis brings together expertise from public health and research areas, and builds on a plethora of knowledge in the arbovirus field. This dynamic interaction of disciplines brings renewed interest in test development and hopes for more advanced and accessible diagnostic testing solutions for ZIKV and other arboviruses of global importance.

Notes

Financial support. This work was conducted by NIH employees in the course of their usual duties without additional funding support.

Supplement sponsorship. This work is part of a supplement sponsored by the National Institute of Allergy and Infectious Diseases (NIAID), part of the National Institutes of Health (NIH).

Potential conflicts of interest. The author reported no conflicts of interest. The author has submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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