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Title: Testing for Zika Virus (ZIKV) Infection in Pregnancy: Key Concepts to Deal with an Emerging Epidemic

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Abstract

Zika virus (ZIKV) is an emerging mosquito-borne (*Aedes* genus) arbovirus of the *Flaviviridae* family. Following epidemics in Micronesia and French Polynesia during the past decade, more recent ZIKV infection outbreaks were first reported in South America as early as May of 2013, and spread to now 50 countries throughout the Americas. Although no other flavivirus has previously been known to cause major fetal malformations following perinatal infection, reports of a causal link between ZIKV and microcephaly, brain and ocular malformations, and fetal loss emerged from hard hit regions of Brazil by October 2015. Among the minority of infected women with symptoms, clinical manifestations of ZIKV infection may include fever, headache, arthralgia, myalgia and maculopapular rash; however, only one out of every four to five people who are infected have any symptoms. Thus, clinical symptom reporting is an ineffective screening tool for the relative risk assessment of ZIKV infection in the majority of patients. As previously occurred with other largely asymptomatic viral infections posing perinatal transmission risk (such as HIV or CMV), we must develop and implement rapid, sensitive, and specific screening and diagnostic testing for both viral detection and estimation of timing of exposure. Unfortunately, despite an unprecedented surge in attempts to rapidly advance perinatal clinical testing for a previously obscure arbovirus, there are several ongoing hindrances to molecular and sonographic based screening and diagnosis of congenital ZIKV infection. These include: (1) difficulty in estimating the timing of exposure for women living in endemic areas, and thus limited interpretability of IgM serologies; (2) cross-reaction of IgM serologies with other endemic flaviruses, such as dengue (DENV); (3) persistent viremia and viruria in pregnancy weeks to months after primary exposure; and (4) fetal brain malformations and anomalies preceding the sonographic detection of microcephaly. In this commentary, we discuss screening and diagnostic considerations which are grounded not only in the realities of current obstetrical practice in a largely global population, but in basic immunology and virology. We review recent epidemiologic data pertaining to risk of congenital ZIKV malformations based on trimester of exposure, and consider side by side with emerging data demonstrating replication of ZIKV in placental and fetal tissue throughout gestation. We discuss limitations to ultrasound based strategies which rely largely or solely on the detection of microcephaly, and provide alternative neurosonographic approaches for the detection of malformations which may precede or occur independent of a small head circumference. This expert review provides information that is of value for

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the: 1) obstetrician, maternal-fetal medicine specialist, midwife, patient and family in cases of suspected ZIKV infection; 2) reviews the methodology for laboratory testing to explore the presence of the virus and the immune response; 3) ultrasound based assessment of the fetus suspected to be exposed to ZIKV with particular emphasis on the central nervous system; and 4) identifies areas ready for development.

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Glossary of Terms

Avidity: the overall binding strength between an antibody and an antigen, which is reflective of both the affinity of the antibody for its epitope and the antibody valency. In general, avidity testing is done with IgG isotype antibodies and lower avidity is seen with more recent infection.

Arbovirus: viruses transmitted by arthropod (including mosquitoes) vectors

Dengue virus (DENV): DENV is the causative virus of dengue fever. Like ZIKV and DENV, CHIKV is a positive-stranded RNA arbovirus of the *Flaviviridae* family, genus *Flavivirus*. Unlike ZIKV, despite well documented exposures in pregnancy, DENV does not cause congenital malformations.

Chikungunya virus (CHIKV): CHIKV is the causative virus of chikungunya. Like ZIKV, DENV is a positive-stranded RNA arbovirus of the *Togaviridae* family, genus *alphavirus*. It too is transmitted by the same Aedes spp. of mosquitos, and similar to both ZIKV and DENV infection causes mild to severe symptoms of fever, rash, arthralgia, and headache. However, unlike ZIKV, it is not known to cause congenital malformations.

Flaviviridae family: The family of viruses to which both DENV and ZIKV belong. Humans and other mammals serve as their natural hosts, and they are transmitted primarily through arthropod vectors such as mosquitos and ticks. Other members of the family include Yellow Fever virus, West Nile virus, and St. Louis encephalitis virus (all of the genus *Flavivirus*), as well as Hepatitis C (genus *Hepacivirus*).

Microcephaly: Acute or chronic slowing in head growth resulting in a small head circumference relative to gestational age and body size, strictly defined as measuring greater than 3 standard deviations (SD, or Z scores) below the standardized population mean. There are multiple causes of microcephaly, including congenital infections (such as ZIKV, CMV, toxoplasmosis, rubella and herpes virus, syphilis, and HIV), chromosomal abnormalities (including both aneuploidy and structural malformations, such as Downs syndrome and microdeletion syndromes), exposure to toxic environmental pollutants and teratogens (such as arsenic and mercury, alcohol, and radiation), and acute trauma with hypoxic-ischemic injury. During the course of the current ZIKV pandemic, different organizations and publications have used a working definition of microcephaly of both -2 and -3 SD to define microcephaly.

NAT (nucleic acid test): generalized term referring to a nucleic acid test (NAT) or nucleic acid amplification test (NAAT), which are molecular based approaches for detecting and quantifying a particular pathogen (virus or bacterium) in a specimen of blood or other tissue or body fluid.

Pandemic: an infectious epidemic which has documented spread across a large region, generally spanning continents.

PRNT (plaque reduction neutralization test): quantification of the titer of neutralizing antibody for a virus. Values are provided as fold higher or lower titers by PRNT.

rRT-PCR (real time reverse transcriptase polymerase chain reaction): the NAT methodology employed currently for ZIKV, rRT-PCR quantitatively monitors the amplification of ZIKV nucleic acid through the creation of complementary transcripts during the PCR, *i.e.* in real-time.

Zika virus (ZIKV): the causative viral pathogen of Zika disease and congenital Zika syndrome.

Introduction. Human infection with ZIKV, an emerging mosquito-borne flavivirus (*Flaviviridae* family, *Flavivirus* genus), has reached pandemic levels in the Americas with at least 50 countries or territories, including Puerto Rico, Florida and Texas, reporting infection over the interval from May 2015 through November 2016 (1). The recently confirmed causal link between ZIKV and fetal microcephaly now places ZIKV among the relatively small list of infections in pregnant women that lead to congenital anomalies. Coupled with the recent pandemic, this has led to unprecedented numbers of pregnant women at-risk for having fetuses with severe abnormalities.

Epidemiology and estimates of congenital ZIKV infection. Previous outbreaks of ZIKV were largely sporadic across Southeast Asia and equatorial African belts, but later spread east resulting in an outbreak in Yap Island in 2007, followed by epidemics in French Polynesia, New Caledonia, the Cook Islands, and Easter Island in 2013 and 2014 (2,3). Until recently Zika viral (ZIKV) illness was thought to be self-limiting, resembling a mild version of dengue (DENV) or chikungunya (CHKV) virus. ZIKV is transmitted via *Aedes* spp of mosquitos, and spread via sexual transmission, vertical (mother to child), and blood transfusions (1). Clinical manifestations of ZIKV infection include fever, headache, arthralgia, myalgia and maculopapular rash, although only one out of every four to five people who are infected manifest symptoms (2-5). Thus, clinical symptoms are not an effective screening tool for the diagnosis or relative risk of ZIKV infection since approximately 80% are likely asymptomatic.

Based on initial reports of relatively mild symptoms accompanying infection, ZIKV was not thought to lead to severe consequences. Exceptions included rare cases of Guillain-Barre (73 out of 28,000 cases) and even rarer instances of perinatal transmission (2 initial cases, with potentially as great as 1% by later estimates) first reported during the French Polynesia outbreak (2,4). However, as ZIKV spread to the Americas in far higher volume (1.3 million autochthonous cases by December 2015), an approximate 20-fold increase in congenital cases of microcephaly with brain and ocular malformations was reported throughout northeast and southeast Brazil (6). Although no other flavivirus is known to cause disseminated fetal neural malformations in humans, worldwide concern for latent viral disease was raised following several case reports demonstrating ZIKV RNA in the amniotic fluid, placenta, and fetal neural tissue weeks to months after initial maternal infection (4; 6-10).

More recently, however, several national and regional cohorts or registries have provided further evidence suggesting that there are multiple clinical manifestations of ZIKV infection in pregnancy. The first of these studies was published as a preliminary description of a prospective cohort of 88 symptomatic gravidae from Rio de Janeiro that had been followed throughout gestation (11), with a further expanded description published in late December, 2016 and inclusive of 134 ZIKV positive women (12). In the preliminary report (11), Brasil and colleagues described that 42 of the 72 symptomatic gravidae who tested positive for ZIKV underwent ultrasound examination, with 29% (12 of 42 ZIKV) demonstrating variable findings on ultrasound, ranging in presumptive severity from CNS lesions with microcephaly, to isolated findings suggestive of placental insufficiency such as fetal growth restriction, or abnormal umbilical artery Doppler velocimetry or amniotic fluid volume (11).

Fortunately, Brasil *et al* (12) published an expanded recent follow-up from this prospective cohort with detailed pregnancy and infant outcomes. Inclusion into this expanded study cohort of 345 women was limited to those presenting to a single clinic in Rio with a rash, and all positive ZIKV cases were defined by testing positive within 5 days of rash development for ZIKV viral nucleic acid by PCR (QuantiTect Probe rRT-PCR; 12) on blood specimens, urine specimens, or both. Of the 345 gravidae with a rash enrolled, 182 were PCR-positive for ZIKV and 163 were PCR-negative. Of the 182 ZIKV-positive, 125/134 had delivery and follow up data; of the 163 ZIKV-negative, 61 were followed through delivery (12). Because only gravidae presenting to the clinic with a rash were enrolled in this cohort study, it is not surprising that ZIKV-negative women were more likely to have positive CHIKV IgM or PCR results (41.7% or 25/60, vs 2.8% or 3/106 tested, $p<0.001$). Interestingly, more than half of ZIKV-positive women presented with acute infection in the second trimester, and ZIKV-negative women were more likely to have used insect repellent (80% versus 60%, $p=0.0006$).

They reported adverse pregnancy outcomes in 46.4% (58/125 after 9 of the 134 gravidae were lost to follow up), including a 7% risk of fetal loss (9/125; 6/9 miscarriages and 3/9 stillbirths) and 41.9% (49/117 live births) with congenital anomalies noted by the first month of life (49/117; ref 12). The rate of adverse pregnancy outcomes including fetal loss with laboratory-documented ZIKV infection was similar and did not significantly

vary by trimester of exposure (55% or 11/20 of pregnancies in the first trimester, 52% or 37/72 in the second trimester, and in 29% or 10/34 of those in third trimester (12). In comparative analysis with the ZIKV-negative pregnancies, a statistically significant difference was observed for elevated risk of adverse pregnancy outcomes in any trimester with ZIKV documented infection (46.4% versus 11.5%, $p<0.001$), emergency Cesarean delivery (23.5% vs 2.5%, $p=0.003$), and evidence of abnormal neonatal and infant findings (41.9% versus 5.3%, $p<0.001$; 12).

Interestingly, Brasil *et al* (12) described that almost all of the neonatal and infant abnormalities detected in ZIKV-positive gravidae affected the central nervous system (CNS) but were not accompanied by microcephaly *per se*. Positive postnatal findings included microcephaly, cerebral calcifications, cerebral atrophy, ventricular enlargement, hypoplasia of cerebral structures, parenchymal brain hemorrhages, and gross findings on postnatal exam. In fact, among ZIKV-positive gravidae a total of 31 of 49 (63%) infants available for follow up displayed one or more of the following: hypertonus, spasticity, limb contractures, seizures, persistent cortical thumb sign or clenched fists, redundant scalp skin (even among normocephalic infants), and abnormal funduscopic or audiology exams (12). Of note, although the data provided is descriptive, the authors found that a number of infants with normal clinical assessments in early infancy had abnormal nonspecific postnatal MRI findings (12). These included diffuse T2 hypersignaling in the peritrigonal posterior areas and less evident in the frontal parietal white matter, with diffusion sequence hyposignaling. These findings are abnormal in infants, and raise concern for cortical tract dysfunction (12).

Although the reports of Brasil and colleagues are limited to symptomatic women from a single pregnancy clinic in Rio de Janeiro, a similar spectrum of congenital findings have been described among women exposed in other regions of the Americas (13). Honein and colleagues (13) have provided recent estimates from a United States CDC and health department registry (US Zika Pregnancy Registry, or USZPR) of 442 completed and registered pregnancies. These U.S. based investigators reported that 6% (26/442, 95% CI, 4%-8%) of maternal ZIKV infections result in congenital birth defects, and reach 11% (9/85, 95% CI, 6%-19%) when exposure was documented exclusively in the first trimester or preconception; no cases of microcephaly or brain malformations were detected unless first trimester exposure was documented to occur (13). However,

gestational age at infections was unknown for 2 of 26 fetuses or infants with birth defects and 27 of the 442 total completed pregnancies; nearly half of all women in the U.S. Zika registry had exposure during multiple trimesters of pregnancy. Of the 26 affected fetuses or infants, 4 had microcephaly but no reported neuroimaging, 14 had microcephaly and brain abnormalities, and 4 had brain abnormalities without microcephaly. Malformations noted included intracranial calcifications, abnormalities of the corpus callosum and cortical formation, cerebral atrophy, ventriculomegaly, hydrocephaly, and cerebellar abnormalities (13). The rate of congenital ZIKV among reported pregnancies did not differ by symptom occurrence or severity (6% in both groups), and asymptomatic registry subjects were just as likely to have an affected infant or fetus as symptomatic subjects (16/271 symptomatic, 95% CI 4%-9%, vs 10/167, 95% CI 3%-11%; reference 13). Interestingly, of the 442 women, 61% (271/442) were asymptomatic and 38% (167/442) were symptomatic (13).

There are several comments pertaining to the U.S. registry report of Honein and colleagues which are worth considering prior to implementing their findings as part of obstetrical clinical counseling or risk assessment. First, registered subjects represented exposure resulting from travel of the subject or their partner to endemic regions besides just Brazil, and include Barbados, Belize, Colombia, Dominican Republic, El Salvador, Guatemala, Haiti, Honduras, Mexico, Marshall Islands, and Venezuela. Second, registry inclusion necessitated laboratory evidence of possible ZIKV infection and required either physician or public health reporting of positive cases. As with any voluntary registry, there is a strong risk of ascertainment bias. Both performance of testing for exposure to ZIKV and entry into the registry may be biased by either symptoms or ultrasound-based detection of anomalies. Similarly, a positive ZIKV test may have precluded additional genomic testing, including chromosomal microarray and karyotypic analysis. In addition, access to and availability of testing likely limited the population denominator in this study, and the human factors related to reporting may have overrepresented the numerator. This possibility for is best realized by the reported rate of 61% of registry subjects reporting symptoms, which is two or three times the anticipated rate (1). Finally, although no birth defects were reported among the pregnancies with maternal symptoms or exposure only in the second trimester (0/76) or third trimester (0/31), ongoing follow up of infants was not reported and there was insufficient data to adequately estimate the purported affected during the latter two trimesters (13). Since there

are multiple reports of normocephalic neonates at birth after second and third trimester ZIKV exposure who subsequently display postnatal brain malformations (11; 14-16), case affect rates arising solely from the current registry ought to be considered preliminary estimates.

In summary of both the Brazilian symptomatic cohort of Brasil *et al.* (11,12) and the U.S. based registry of Honein *et al.* colleagues (13), tremendous gratitude ought be extended to these investigative teams for their Herculean efforts aimed at collecting, characterizing, and detailing broad and robust perinatal clinical outcomes in the year since the association between ZIKV and fetal malformations was first largely recognized. However, these high impact reports are limited by hindrances inherent to descriptive cohorts and estimates of relative or absolute risk of congenital ZIKV malformations in any trimester of pregnancy remain preliminary. All counseling should be accordingly framed with appropriate degrees of uncertainty. This would include acknowledging a persistent but not currently quantifiable attributable risk for congenital ZIKV malformations following infection at any point during gestation, and a need for postnatal follow up. Moreover, until population based studies are completed with universal testing of both asymptomatic and symptomatic women at risk of exposure, the true attributable risk estimate cannot be determined with any degree of confidence.

Given the challenges in estimating the likelihood of congenital malformation following perinatal ZIKV exposure, we are left to question to what extent the current ZIKV pandemic will affect global health burden in the coming decades. In the absence of ZIKV exposure in the population, microcephaly occurs in approximately 7 per 10,000 births (13). Assuming an estimated attack rate of ZIKV as high as 73% (as occurred on the island of Yap; 5), the burden to both individual families and society in the face of a burgeoning pandemic is potentially staggering, even if the risk of microcephaly or congenital brain malformation is as low as 0.1% to 11% among symptomatic gravidae. In light of such a potential burden and need for accurate risk estimates, the importance of accurate and predictive diagnosis is readily evident.

Who should be tested and how? The first step in potentially diagnosing congenital ZIKV infection is recognition of who should be tested. Since the majority of infected patients will have no or mild symptoms, clinical screening is not a reliable tool. There are two key risk factors that make pregnant women eligible for testing: those either with a personal history of exposure risk (either traveling to or residing in an endemic area)

OR sexual contact with a partner bearing the same exposure risk. Recognition of those at risk for ZIKV is therefore predicated on screening patients for regional travel and residence, and sexual history, two areas commonly neglected in standard medical visits. Use of prompts within the electronic medical record may aid in screening.

For those women who have resided in or traveled to a Zika endemic area, the CDC recommends testing inclusive of 8 weeks *prior* to conception and throughout gestation. This recommendation is based on a doubling of the maximal incubation time in non-pregnant women (3 to 14 days) plus intervals of viremia (2-10 days; ref 1) and viruria (up to 14 days) (references 1,17-19). These rough estimations were empirically demonstrated in a non-pregnant subject case report from our group documenting viral shedding in the vaginal mucosa for two months after symptomatic infection (17). In addition, women whose male sexual partners have traveled to areas with active ZIKV transmission have a risk of viral acquisition for 6 months (1, 17-19). However, the exact duration of potential transmission via sexual exposure is currently unknown, although ZIKV RNA has been discovered in semen for up to 188 days after illness onset (18). Important pitfalls to these guidelines include the potential for prolonged viremia and viruria noted in pregnant women (20-22).

Why should we test and not just screen for microcephaly with ultrasound? Molecular diagnostics for viral pathogens and estimation of risk. There are two primary reasons while screening for microcephaly is insufficient. First, there are multiple malformations which may not entail microcephaly, including intracranial calcifications, abnormalities of the corpus callosum, and cerebellar abnormalities (13). Second, in instances of abnormal cortical formation, cerebral atrophy, and cortical neuronal agenesis, neuronal death will occur over time and microcephaly will be observed with age (11; 14-16). Fortunately, over the past two decades, clinical laboratory medicine has witnessed an unprecedented capacity in the ability to rapidly test for infectious pathogens. It was not long ago when diagnosis relied on weeks of viral cultures which were often low or nil yield, while today's current clinical lab medicine and pathology practice allows for highly sensitive and specific rapid molecular diagnostic testing. Ergo, we are notably handicapped when we cannot reliably identify a potential viral pathogen or establish exposure and immunity with serologic testing. In many circumstances diagnostic testing is utterly and fundamentally necessary to both personalized clinical management and public

health control measures. This is no truer than when a viral pathogen will result in a devastating perinatal transmission, yielding a potentially severely compromised infant faced with lifelong disability. In the short term, diagnoses are important for decisions of pregnancy continuation. However, in the longer term, accurate and early diagnosis becomes the cornerstone of developing targeted and efficacious interventions, and estimating the natural history of an infection to give truly informed risk estimates.

Currently available laboratory-based testing for ZIKV. There are several limitations to currently available testing for ZIKV. To illustrate these limitations, consider the following hypothetical clinical scenario. 'Ms. Jones' is a 35 year old G2P0010 who resides in Texas, but works part-time in Brazil. Her husband resides and works full time in Brazil. She comes to see you at 16 weeks gestation, after she completed a 4 month interval working in Rio with frequent travel to the Northern provinces. You have performed an ultrasound which shows borderline bilateral ventriculomegaly at 11 and 12 mm, with overall estimated fetal weight at the 24th centile and a head circumference and biparietal diameter at the 15th centile. Per the CDC algorithm (1,19), she should be eligible for IgM testing up to 12 weeks from entry back into the U.S. However, what if she were actually infected at 4 weeks gestation? Her IgM might be negative or indeterminate as a result of natural titer waning. In the absence of an IgG isotype serologic test, a negative or indeterminate IgM titer would potentially be interpreted falsely as negative. Knowing these limitations, what are the testing options for this patient? Would rRT-PCR testing be helpful? When should each test be performed? How should we interpret our testing results, and what are the current limitations to these interpretations? In the following sections we discuss testing options, **Tables 1** and **2** provide summary guidance.

The diagnosis of ZIKV infection currently relies on detection of viral RNA via real-time reverse transcriptase polymerase chain reaction (rRT-PCR) or identifying an IgM serologic response (1,17-19). Given testing limitations, diagnosis and patient counseling are inexact with poor predictive sensitivity (rRT PCR) and antibody cross-reactivity (IgM). Because few experience symptoms and travel or residence in endemic areas frequently spans weeks if not months, the exact timing of exposure is often unknown or cannot be reliably estimated. This is particularly a concern when the serologic testing relies upon an initial response antibody, such as the pentavalent IgM isotype antibody. As a consequence of isotype class switching (20), the first

antibody serologically to rise will be the IgM and given its pentavalency is often cross reactive with other viral strains and family members; whether this is true for ZIKV and other related flaviviruses is not yet known. IgM titers may initially be detectable between 4 and 14 days (1,17-19), but typically not until 10-14 days. The titers will start to measurably wane by 10-12 weeks. Subsequently, higher avidity IgG isotypes will appear and remain positive for longer periods of time and thus provide ongoing immunity (20). Similarly, possibly paralleling the variation in clinical symptom severity, even currently symptomatic patients may not test positive in either serum or urine by rRT-PCR (1,17-19).

rRT-PCR testing. The incubation period for ZIKV is 3-14 days, and in non-pregnant subjects' serum viremia lasts for as few as 2 days to as great as 10 days (1). Longer periods of viremia and viruria have been observed during pregnancy presumably due to fetal-placental infection (21,22). Virus persists on average 2 weeks longer in urine and testing for viruria has been shown to improve detection rates (23). Since the majority of patients (80%) with ZIKV infection are asymptomatic (1), identifying patients during the viremic or viruric stage is challenging. Current CDC guidelines recommend testing serum or urine by rRT-PCR when a patient presents within 14 days of their last potential exposure to ZIKV, or within 14 days of symptoms (1,18,19). Their last potential exposure can be either the date of sexual contact with someone at risk for ZIKV, or last date of travel to a ZIKV endemic region. Strictly adhering to this testing algorithm would by definition miss prolonged viremia, which may be a surrogate of fetal-placenta infection (21,22).

Other body fluids where ZIKV RNA (but not necessarily infectious virions *per se*) has been detected include saliva, semen, breast milk, vaginal and cervical mucous (1). These niches potentially serve as latent portals for continuing infectivity of ZIKV, either to the mother or the fetus. Fortunately, horizontal transmission has only been documented via sexual intercourse (both male to female, female to male, and amongst same sex-partners) and blood transfusions, both in reported infrequent numbers (1,17-19,21-25). However, the possibility of transmission from contact with other infected body fluids is certainly a looming concern (24). Persistence of ZIKV RNA in other body compartments also provides an opportunity to expand testing options (17). The level of ZIKV is higher in semen than urine, saliva or serum and is detectable for longer periods of time after acute infection (25). This may be of particular interest in family planning. Saliva and urine testing are potential fluids

for detection of ZIKV RNA and may be of interest in certain populations, such as children and neonates (where drawing blood is difficult) or in settings of limited resources when the additional cost and availability of phlebotomy services are burdensome (26). Detection of ZIKV nucleic acid has been observed in breast milk but no documented cases of transmission have occurred as of yet (27). A recent case report from our own institution demonstrated persistence of ZIKV in the red cell compartment for up to 81 days after symptoms, 73 days longer than serum, suggesting that the use of whole blood instead of serum may increase the sensitivity of ZIKV detection, particularly in the asymptomatic patient (17).

Serologic Testing. Serologic testing is recommended 4 or more days after onset of clinical illness or ≥ 14 days from the last potential exposure in asymptomatic patients. An IgM capture enzyme-linked immunosorbent assay (MAC-ELISA) is the only FDA approved serologic test and can be performed on serum and cerebrospinal fluid. Evidence from the serologic response to other flaviviruses, particularly Dengue (DENV) and West Nile virus, suggests that IgM may be present up to 3 months post-exposure; in some patients with West Nile encephalitis, IgM antibodies were detectable more than 1 year after infection (28-31), with one study showing IgM antibodies persisting up to 8 years post infection (32). At present, serologic testing for ZIKV with IgM is currently recommended up to 12 weeks post-exposure as serologic waning and trough intervals are not yet known. IgG antibodies to ZIKV develop shortly after IgM antibodies and are thought to confer lifelong immunity with higher avidity. For a brief interval from September through August at least one commercial laboratory offered ZIKV IgG, but it is presently not available. Undoubtedly availability of IgG serologies with potential avidity testing would be advantageous.

In addition to waning titers of IgM isotypes, there is often serological cross-reactivity with other flaviviruses in patients who have had a recent or prior flavivirus infection, particularly DENV. This complicates diagnosis, particularly since ZIKV is emerging in areas where DENV is endemic. Currently, serologic differentiation and confirmation relies upon a more cumbersome and less available testing method, the plaque reduction neutralization test (PRNT). The PRNT identifies virus-specific neutralizing antibody titers to various related flaviviruses. Use of the PRNT necessitates contextual estimations, and a PRNT fold-titer of >10 with a competing flavivirus antibody titer (for example, Dengue; DENV) of fold-titer of <10 , would suggest recent

infection with ZIKV the past 3 months (**Table 2**). However, what often results are PRNT estimates with dual elevations, such as ZIKV PRNT fold-titer of >10 and DENV PRNT fold-titer of >10 is an acute flavivirus infection, type indeterminate, and may represent either a recent ZIKV or DENV infection, or a co-infection (see Tables 1 and 2). To date, PRNT testing is only offered through the CDC and there is ongoing high demand with prolonged turn-around times.

In summary, a negative ZIKV IgM test may represent either a patient who is not at risk, is too early in the course of infection and has not yet seroconverted, or who had infection with ZIKV and has already seroconverted to IgG with a physiologic waning of their IgM titer (**Tables 1 and 2**). A positive IgM test indicates either (1) a false positive (2) acute ZIKV or (3) another acute flavivirus infection, including co-infection with ZIKV. In patients experiencing symptoms for less than 2 weeks, lack of seroconversion needs to be evaluated by rRT-PCR testing on both a serum and urine specimen as recommended by the CDC. With the development and dissemination of ZIKV-specific IgG serologic and avidity testing, the potential for expanded serologic testing will be enabled.

Amniocentesis. A growing body of literature suggests infants with confirmed fetal infection are at increased risk of intracranial abnormalities (33). In the setting of a positive ZIKV IgM with positive PRNT, positive rRT-PCR in maternal serum or urine, or with ultrasound abnormalities, amniocentesis to detect ZIKV RNA via rRT-PCR should be considered to evaluate for vertical transmission (**Table 1**). However, the sensitivity, specificity, positive and negative predictive value of rRT-PCR on amniotic fluid is unknown, and these potential benefits and limitations should be discussed openly. In cases where ultrasound abnormalities are detected, it is our clinical practice to also perform CMA (34) and test for other congenital pathogens in the setting of ultrasound abnormalities.

Ultrasound based fetal assessment. The CDC, American Congress of Obstetricians and Gynecologists (ACOG; 35), the Society for Maternal-Fetal Medicine (SMFM; 36) and the International Society of Ultrasound in Obstetrics and Gynecology (ISUOG; 37), have recommended ultrasound evaluation for fetal anomalies in pregnant women who have been infected or potentially exposed to ZIKV. Paramount to these recommendations have been the detection of microcephaly using ultrasonography (19; 35-37). Microcephaly is

defined as a head circumference (HC) >3 standard deviations below the mean for estimated gestational age, with fewer false positives the further the HC falls from the mean.

A causal criteria between ZIKV and microcephaly was acknowledged by the CDC in April 2016, and multiple other congenital anomalies have been associated with congenital ZIKV (38,39). Thus, it is possible that microcephaly may represent an extreme endpoint in the spectrum of cerebral cortical hypoplasia and tissue loss (**Table 3**). Some recent studies have indicated that ZIKV-associated congenital malformations can occur after infection in any trimester, or even after birth in infants with normal head circumference at delivery (12, 14-16) a finding which differs from the typical pattern seen in other congenital infections. It is possible that this is the result of the placenta functioning as a reservoir for ZIKV replication, a pattern which differs from most other congenital infections (40). However, other studies indicate the risk maybe predominantly in the first trimester (11,41). Thus, microcephaly is likely the end result of cortical agenesis and brain volume loss, rather than a primary consequence of fetal viral infection or calvarium destruction. Ergo, screening for microcephaly as initial evidence of fetal infection is likely too little too late.

The congenital ZIKV syndrome portends a constellation of findings, inclusive of intracranial abnormalities, which may or may not include microcephaly (**Table 3**) (42). In a review of 438 symptomatic subjects with rash or suspected fetal abnormalities, 17 had confirmation of fetal infection based on either positive amniotic fluid, cord blood, or neonatal brain tissue at autopsy, and 28 had presumed infection based on CNS findings. Of these subjects, almost all had intracranial abnormalities with multiple instances of intracranial abnormalities with normal head circumference measurements (33). Similarly, a second observational study has shown that ventriculomegaly and microcephaly progress as pregnancy advances (43), which would explain the observation from the French Polynesian outbreak that first trimester infection portends a stronger association with microcephaly than infection later in pregnancy (41), a finding recently confirmed in the US Zika Registry (13). We now know that ZIKV infection as late as 27 weeks causes continued CNS damage into the first 6 months of life despite a normal head circumference during pregnancy and at delivery, suggesting that the neurologic damage caused by ZIKV is a continuum that may begin *in utero* but does not necessarily end with delivery (12, 14-16;45). Limitations to these studies include the small number of cases of microcephaly (n=8 in

French Polynesia), and small number of women who had delivered by the time of publication (n=8 deliveries in Rio de Janeiro) with no follow-up data after delivery and theoretic estimates of risk based on mathematical modeling (4,7,10).

Given the limitations of findings by ultrasonography alone for the evaluation of congenital ZIKV infection (**Table 3**), we view sonographic screening as an additional clinical tool (potentially aiding in our diagnosis) which could be complimentary to molecular diagnostics. A recent Society for Maternal-Fetal Medicine (SMFM) statement recommended that if the HC by prenatal ultrasound is >2SD below the mean, a careful evaluation of the fetal intracranial anatomy is indicated. If the intracranial anatomy is normal, SMFM recommends follow-up scans in 3-4 weeks (36). These recommendations acknowledge that fetal brain malformations and findings of congenital ZIKV disease will occur independent of (and in fact may precede) strictly defined fetal microcephaly at >3 SD below the mean (9,35-37,44,45). Based on these recommendations by SMFM and other professional entities, a further discussion regarding fetal neurosonographic imaging is warranted.

Fetal brain imaging in cases of suspected congenital ZIKV infection. An evaluation of intracranial anatomy requires the use of conventional sonographic planes including the transventricular, transthalamic and transcerebellar views and the brain parenchyma to evaluate for structural anomalies as well as calcifications. Given these considerations in total, our current practice at Baylor College of Medicine is to perform neurosonographic (neurosonology) screening in at-risk women at the time of their mid-trimester fetal anatomic survey, and every 4-6 weeks thereafter (36). Because fetal brain malformations may be either incident to or causative of microcephaly, we do not predicate indication for neurosonography (neurosonology) on presence or absence of microcephaly but rather use parallel testing results and at-risk estimations as our indication (**Figures 3 and 4, Table 3**). Neurosonography (neurosonology; 46) is best performed via transvaginal sonography if the fetus is in a cephalic presentation, but it can also be performed using the transabdominal approach. Transventricular, transthalamic and transcerebellar planes should be obtained in the axial view. Midsagittal and parasagittal planes should be obtained in the sagittal view and transcaudate and transcerebellar planes should be obtained in the coronal views. In each view, brain development and the integrity of its anatomic structures should be assessed (47-49). In addition, the head circumference, biparietal

diameter, ventricular widths at the level of the anterior and posterior horns, third ventricle, transcerebellar diameter, posterior fossa, vermian height, and callosal length should be measured (**Figures 3 and 4**; 46). These measurements should be referenced to previously published normality values considering the gestational age at the time of the exam (50-53). For fetal head circumference, SMFM (36) advocates for utilization of Chernevak's reference values (54), while ISUOG advocates for use of Intergrowth 21 reference values (55). Fetal brain sulcation should be assessed in detail, with consideration of the cortical fissures which would normally be observed in subsequent gestational age windows (**Figure 4**; 56,57). Fetal MRI can also be performed, either in lieu of or as an adjunct to neurosonology, to better characterize intracranial abnormalities. Fetal MRI may be particularly helpful in the assessment of potential cortical and brain sulcation abnormalities where detection and differentiation is limited by using ultrasound imaging alone (33). Referral to a provider or center with experience in neurosonography (neurosonology) and fetal MRI should be considered.

How do we counsel women with laboratory based evidence of recent ZIKV infection?

Unfortunately, at this time the main benefit of ZIKV screening and testing lies in patient counseling, with notable currently present degrees of uncertainty in ascribing absolute or attributable risk estimates. While there is not a current treatment option for maternal or fetal ZIKV infection, this is not dissimilar from antenatal genetic testing, and therefore the value of diagnosis should not be undervalued. Moreover, although we still do not know the true risk of congenital ZIKV with maternal infection, there are several studies from which we can provide both risk and uncertainty estimates to our patients. First, in the United States asymptomatic women may be at similar risk to symptomatic women (13). There appears to be a wide estimated occurrence and gestational ages of susceptibility reported, ranging from <1-29% (11-16,41). Whether infection in the first trimester has a higher rate of congenital ZIKV syndrome and microcephaly than infection in other trimesters is uncertain, but risk being exclusive to windows of exposure in the periconception and first trimester is highly improbable or reported not to be true (5,8,10-13,35,36,38-43,45,49,58-60). In other words, at the present there is no trimester known to be absent of risk for congenital ZIKV syndrome.

Regardless of our present inability to accurately or precisely ascribe relative or attributable risk for congenital ZIKV syndrome, there are several grounded statements which can be currently used when counseling women

and their partners. First, all evidence to date suggests that just as with other vertical transmissions, only a minority of women with any ZIKV exposure (symptomatic or asymptomatic) will be at risk for congenital anomalies at birth (5,8,10-13,35,38-43,49,58-60). Second, we still do not have adequate data to inform counseling regarding the incidence of late congenital infection; therefore long term follow up of neonates is essential (12,13,38-42;45). Lastly, women with an amniocentesis rRT-PCR positive for ZIKV likely have an appreciably greater risk of congenital ZIKV infection, and the neurocognitive effects are likely to be significant (27). However, due to potential limitations of detection in dilute amniotic fluid volumes, absence of ZIKV by rRT-PCR cannot be considered a reliable means of “ruling out” congenital ZIKV infection.

Regulatory challenges. The mainstay of current diagnostic capabilities include rRT-PCR and IgM serology with a heavy reliance on PRNT for serologic testing interpretation and confirmation (**Table 1**, **Table 2**). Missing elements include IgG serologies and avidity testing. Moreover, access to timely diagnosis is limited by regional testing capacity and burden of testing. As more regions in the U.S. become endemic, this burden of testing and need for improved sensitivity and specificity will only increase. Expansion of testing modalities to both hospital-based and commercial laboratories, alongside city, county and state health departments, will be crucial to capacity expansion. FDA regulations in an emerging pandemic are decidedly important, as both test precision and accuracy are of utmost importance. However, these concerns must be balanced with diminished regulatory burden to enable the development of crucially important timely testing in the face of a congenital infectious pandemic.

Knowing what we don't know, and prioritizing what we need to learn. Despite the advances in understanding the natural history and risk of ZIKV during the past year, including adverse pregnancy outcomes and long-term neurologic effects, there are a multitude of questions that remain unanswered which impede our ability to provide adequate counseling and timely pregnancy management.

First, we do not know risk estimates for fetal infection or CNS abnormalities in the setting of either maternal or amniotic fluid infection as measured by rRT-PCR. Second, there are multiple strains of ZIKV and the majority of ZIKV strains isolated in the Americas are of Asian lineage, and all are associated with fetal congenital infection. However, it is unknown if African strains convey similar risk which was previously under reported, or

if viral mutations have imparted the capacity for congenital malformations. Similarly, it is unknown if infection with one ZIKV strain will confer lifelong immunity and protection against all ZIKV strains (58). Clarifying these distinct risks and potential immunity may be important in vaccine development, particularly if implemented in pregnant or reproductive-aged populations. Third, the exact mechanism of ZIKV entry into the fetal compartment has not been defined. Multiple lines of evidence suggest that placental cells, including placental macrophages, are permissive to replication (40,58). However, the implications of these findings to fetal infection, particularly as a portal of delayed entry, are currently unknown (40,59,60). Fourth, maternal viremia and viruria has been observed to persist much longer during pregnancy than what is observed in the non-pregnant population and it remains unknown if this observation is predictive of pregnancies at highest risk of fetal abnormalities or a confounder that results from the pathophysiology and immunologic changes during pregnancy. Fifth, information regarding consequences of neonatal infection is lacking. Since significant CNS growth and development continues into the third trimester, this may be an age group susceptible to ZIKV infection unique to what has been observed with ZIKV infection later in life. Long-term data regarding late neurologic complications from maternal ZIKV infection during pregnancy are crucial for answering these questions. Similar to the long-term cardiovascular comorbidities observed in women who experienced preeclampsia during pregnancy, it remains unknown if ZIKV portends a similar adverse neurologic risk profile. Lastly, testing options need to be expanded and interpretation as to risk of fetal congenital infection needs to be clearly understood. The introduction and validation of IgG testing would further improve determining patients at-risk and the timing of infection. Further exploration into the incidence and prognostic value of prolonged maternal viremia is important, but likely will not occur if patients are only tested within 2 weeks from exposure. Quantitative measures, similar to HIV viral load, may be able to predict latent infection and subsequent risk of fetal infection. Similarly, detection and persistence of positive and negative strand ZIKV RNA by single molecule fluorescent *in situ* hybridization (FISH) technology in different tissue compartments, akin to active viral replication, may act as a surrogate to viral load quantification that can be used in appropriate resource settings.

Future prevention and interventions. Creating therapeutic interventions against maternal-to-child-transmission of ZIKV infection poses a number of challenges. Recent findings that asymptomatic maternal

infection can and will pass ZIKV to the unborn fetus at approximately the same rate as symptomatic (13) suggest that any efficacious population-based approaches would likely rely mostly on vaccinating women of reproductive age prior to conception. While several prototype ZIKV vaccines are under development and in clinical trials, especially at the NIAID, NIH (61), we can anticipate that a number of scientific and regulatory hurdles could ultimately slow down an advance towards licensure (62). Among them are the absence of known correlates of protection and potential safety concerns, including the possibility of vaccine-induced Guillen Barre Syndrome and the general safety and ethical issue surrounding the vaccination of pregnant women or women who plan to become pregnant in the near future. Traditionally such populations have presented a "high bar" in terms of vaccine regulatory science. Therefore, it is unclear whether we will have a safe and effective vaccine against ZIKV anytime soon. Similarly, several recent reports have suggested use of existing drugs with potential fetal therapeutic potential (63-65). These range from drugs with well-characterized safety profiles in pregnancy (*i.e.*, erythromycin and niclosamide; 63,64) to the use of so-called orphan FDA approved drugs with therapeutic potential but unknown fetal and maternal risk (*i.e.*, caspace inhibitors and the 5-HT3 antagonist palonosetron; 63,65). Given ethical and regulatory hurdles with fetal and pregnancy related research in the U.S. and elsewhere, human clinical intervention trials are likely a long ways off. Ergo, the ongoing development of primate ZIKV congenital infection models are of paramount importance, as they will provide crucially important preclinical data.

Conclusion. In a mere 18 months, ZIKV has rapidly spread to more than 50 countries, including the United States, leaving in its wake devastating fetal and long-term neurologic consequences. The push for precise risk estimates and testing of desperately needed preventative vaccine and therapeutic options necessitates highly sensitive and specific molecular antenatal diagnostic testing and parallel sonographic screening. Once these methodologies are readily available, large prospective, population-based observational and therapeutic trials will be both necessary and sufficient to estimate congenital risk with ZIKV infection and develop effective interventions for eradication or prevention. As evidenced by its spread north into Florida and Texas in recent months, the increasing burden to both obstetrical providers and our laboratory medicine colleagues will only increase and we need to be armed with the diagnostic tools necessary to care for our patients in our local and global community.

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Figure legends

Figure 1. Schematic map of the United States and territories with reported endemic and non-endemic cases (A, current as of December 2016), alongside pictorial images of the *Aedes aegypti* mosquito (B) and ZIKV particles (C). In image C, the transmission electron microscopy (TEM) was produced in black and white, with post processing digital colorization. Shown in green are cytoplasmic and nuclear elements of host Vero E6 Green Monkey kidney epithelial cells inoculated with a purified and passaged 1947 Ugandan ZIKV strain. Shown in purple are 40 nm diameter flavivirus particles with a characteristic dense core and outer envelope. TEM images of fixed cells were obtained after purified ZIKV was used to inoculate Vero E6 cells *in vitro*. These images were obtained from the CDC public domain image archives. Images are reproduced with the expressed permission of Dr. Cynthia Goldsmith and the CDC.

Figure 2. *In situ* hybridization (ISH) of ZIKV in human fetal brain and placental tissue. **Left panel:** Localization of ZIKV RNA by *in situ* hybridization in brain tissues from infants with microcephaly. A) ISH with use of antisense probe. ZIKV genomic RNA (red stain) in cerebral cortex of an infant (case-patient no. 66, gestational age 26 wk). Original magnification $\times 10$. B) ISH with use of sense probe. Serial section showing negative-strand replicative RNA intermediates (red stain) in the same areas shown in panel A. Original magnification $\times 10$. C) ISH with use of antisense probe. Higher magnification of panel A, showing cytoplasmic staining of neural (arrowheads) and glial cells. Original magnification $\times 20$. D) ISH with use of sense probe. Higher magnification of panel B, showing cytoplasmic staining of neural and glial cells (arrowheads). Original magnification $\times 20$. E) ISH with use of antisense probe. Localization of negative-strand replicative RNA intermediates in neural cells or neurons (red, arrowheads) of another infant with fatal outcome (case-patient no. 67, gestational age 27 wk). Original magnification $\times 40$. F) Immunostaining of neurons (arrowheads) with use of antibodies against neuronal nuclei in a serial section. Original magnification $\times 40$. G) Hematoxylin and eosin stain showing cortical neural cells in a serial section. Original magnification $\times 40$. H) Immunostaining of glial cells (arrowheads) with use of glial fibrillary acidic protein antibody in the same case. Original magnification $\times 40$. ISH, *in situ* hybridization. **Right panel:** Localization of ZIKV RNA by ISH in placental tissues of women after spontaneous abortion. A) ISH with use of antisense probe. ZIKV genomic RNA localization in placental chorionic villi, predominantly within Hofbauer cells (red stain, arrows), of a case-patient who had spontaneous abortion at 11 wk gestation (case-patient no. 56). Original magnification $\times 10$. B) ISH with use of sense probe. Serial section showing negative-strand replicative RNA intermediates (red stain, arrows) in the same cells shown in panel A. Original magnification $\times 10$. C) Hematoxylin and eosin stain of placental tissue of a case-patient who experienced spontaneous abortion at 8 wk gestation (case-patient no. 47). Original magnification $\times 20$. D) Immunostaining for CD163 highlighting villous Hofbauer cells in a serial section as seen in panel C. Original magnification $\times 63$. E) ISH with use of antisense probe. ZIKV genomic RNA as seen in a serial section from the same case-patient as in panel C, showing staining within Hofbauer cells (red stain, arrows) of placental chorionic villi. Original magnification $\times 40$. F) ISH with use of sense probe. Serial section showing negative-

strand replicative RNA intermediates (red stain, arrows) in the same cells as shown in panel E. Original magnification $\times 40$. G) Hematoxylin and eosin stain from the same case-patient as in panel C, showing inflammatory cell infiltrates in maternal side of placenta. Original magnification $\times 63$. H) ISH with use of sense probe. Negative-strand replicative RNA intermediates (red stain, arrows) in inflammatory cells in a serial section. Original magnification $\times 63$. ISH, *in situ* hybridization. These images have been reproduced with the expressed permission of the author and publisher. Bhatnagar J, Rabeneck DB, Martines RB, Reagan-Steiner S, Ermias Y, Estetter LBC, et al. ZIKV RNA replication and persistence in brain and placental tissue. *Emerg Infect Dis*. 2017. DOI: 10.3201/eid2303.161499 https://wwwnc.cdc.gov/eid/article/23/3/16-1499_article.

Figure 3. Axial sonographic views recommended by ISUOG to perform a targeted CNS scan in order to evaluate the fetal brain (42). Images A,B, and C are axial views; the dashed red lines on images A and C aligns to the Sylvian fissure, while the dashed red line in image B aligns to the parietoccipital fissure. Image **A** corresponds to the transthalamic plane, where measurement of head circumference and biparietal diameter should be performed; the blue dashed line is placed on the location for optimal measurement of the third ventricle width. Image **B** is the transventricular plane, where measurements of fetal ventricular atria width should be performed (blue dashed line). Image **C** corresponds to transcerebellar plane, where measurement of the posterior fossa or cisterna magna and cerebellar width should be performed (43). Images **D** to **G** are key views for neurosonographic (neurosonology) imaging aimed at detection of potential abnormalities detected in the sagittal and coronal planes. Images **D** and **E** are obtained from sagittal views, where **D** is the midsagittal plane view used to assess and measure the height of the corpus callosum (dashed yellow line) and cerebellar vermian (dashed blue line). Also shown in image D is the cingulate fissure (dashed red line). Image **E** represents the parasagittal plane views and can assess brain parenchyma and the ventricles. Both **F** and **G** images are obtained in coronal planes, where **F** represents the transcaudal plane, where the anterior horns of the ventricles (dashed yellow line) and the size of the subarachnoid space (measured by the sinocortical and craniocortical spaces) can be assessed. **G** arises from the transcerebellar plane, and optimally assesses the calcarine fissure (dashed red line) and cerebellum. Abbreviations: CSP: Cavum septum pellucidum; IHF: Interhemispheric fissure.

Figure 4. Representative images from several latter second and early third trimester fetuses affected by congenital ZIKV infection, with axial, sagittal, and coronal views in similar planes to Figure 3 with landmarks in white texts; abnormal findings are highlighted in **off-white bold text**. Images A and B are axial views: Image A shows the dilation of the ventricular system at the level of the posterior horns corresponding to the transventricular plane. The parietoccipital fissure is absent, and there is evidence of diffuse parenchymal thinning with linear calcifications located at the white matter-gray matter (WM-GM) junction (off-white arrows). The subarachnoid space and posterior horns ventricular system are markedly dilated (off-white asterisks). Image B corresponds to the transcerebellar plane. There is a significant dilation of the entire ventricular system as shown by an enlarged third ventricle, and dilated anterior and posterior horns (off-white asterisks). There is

some degree of brain parenchymal thinning with coarse calcifications at the level of the basal ganglia (off-white arrow). Images C, D and E represent the sagittal views: Image C shows an abnormally thin and short corpus callosum in the midsagittal plane (off-white line). While the brain stem and cerebellar vermis have a normal appearance, punctiform calcifications are seen in the frontal and temporal lobe (off-white arrow). Images D and E represent parasagittal views of the fetal brain showing linear and coarse calcifications located on the WM-GM junction and the basal ganglia (off-white arrows). There is evidence of an enlarged ventricular system with thinned brain parenchyma and enlarged subarachnoid space (off-white asterisks). Additionally observed are signs compatible with a delayed sulcation based on the abnormally smooth cortical surface for third trimester fetuses. Images F, G, and H are coronal views: Images F and G show the transcaudal plane, with enlarged anterior horns (off-white asterisks), parenchymal calcifications located at the WM-GM junction and basal ganglia (off-white arrows). Periventricular cysts are seen above and below the anterior horns (off-white arrows). Image H shows a transcerebellar plane with enlarged subarachnoid space and significant ventricular dilatation (off-white asterisks) and accompanying parenchymal thinning. Parenchymal calcifications are seen in the WM-GM junction (off-white arrows).

Table 1. Available ZIKV testing modalities

Test Category		Specimen sources	Timing of first positive ¹	Duration of positive test ¹	Limitations	Interpretations of positive tests
Viral Serology	IgM	Serum CSF	4 days in symptomatic 7-14 days weeks in asymptomatic Unknown	12 weeks Unknown	<ul style="list-style-type: none"> Cross-reactivity with other flaviviruses High false-positive rates Risk for false-negative due to delayed seroconversion or titer waning 	PRNT is needed as follow up test <ul style="list-style-type: none"> False positive Recent ZIKV infection Acute other flavivirus infection (cross reactivity)
	IgG	Serum	7-14 days ²	>12 weeks	<ul style="list-style-type: none"> Currently not available for clinical use 	Other flavivirus infection >12 weeks (cross-reactivity) <ul style="list-style-type: none"> ZIKV Infection >2 weeks, if IgM negative then likely >12 weeks
	PRNT	Serum	With positive serologic testing		<ul style="list-style-type: none"> Only available through the CDC Long turn around time 	See Table 2
Viral Nucleic Acid Testing (NAT)	rRT-PCR	Serum Blood Urine CSF Tissue Amniotic fluid	0-7 days 0-7 days 0-14 days unknown unknown unknown	5-14 days* 5-14 days* 14 days* unknown unknown unknown	<ul style="list-style-type: none"> Prolonged viremia possible and poorly understood <p>*Prolonged viremia and viruria noted in pregnant women and neonates</p>	Recent Zika infection
Ultrasound		Amniotic fluid Biometry Neurologic Extremities	Variable	Once present, appears progressive	<ul style="list-style-type: none"> Poor specificity of findings in isolation or in constellation 	When performed in isolation, cannot distinguish anomaly cause. When performed in conjunction with amniocentesis with chromosomal microarray (CMA) or other infectious pathogen testing, improved specificity; see Table 3

¹Based on current information²Extrapolated from West Nile IgG (24,25)

Table 2. Interpretation of Serologic Results*

IgM ELISA testing	Result	Interpretation
ZIKV IgM	Detected	No evidence of Zika or Dengue infection, false positive IgM
ZIKV PRNT	Not detected	
DENV PRNT	Not Detected	
ZIKV IgM	Positive or equivocal	Recent Zika virus infection
DENV IgM	Positive or equivocal	
ZIKV PRNT	≥ 10	
DENV PRNT	<10	Recent Dengue virus infection
ZIKV IgM	Positive or equivocal	
DENV IgM	Positive or equivocal	
ZIKV PRNT	<10	
DENV PRNT	≥ 10	
ZIKV IgM	Inconclusive in one assay AND inconclusive or negative in the other	Recent flavivirus infection; specific virus cannot be determined
DENV IgM		
ZIKV PRNT	≥ 10	
DENV PRNT	≥ 10	Evidence of Zika virus, timing cannot be determined
ZIKV IgM	Inconclusive in one assay AND inconclusive or negative in the other	
DENV IgM		
ZIKV PRNT	≥ 10	
DENV PRNT	<10	Evidence of Dengue virus, timing cannot be determined
ZIKV IgM	Inconclusive in one assay AND inconclusive or negative in the other	
DENV IgM		
ZIKV PRNT	<10	
DENV PRNT	≥ 10	Evidence of flavivirus infection, specific virus and timing of infection cannot be determined
ZIKV IgM	Inconclusive in one assay AND inconclusive or negative in the other	
DENV IgM		
ZIKV PRNT	≥ 10	
DENV PRNT	≥ 10	

ZIKV: Zika virus; DENV: Dengue virus; PRNT, plaque reduction neutralization tests, with units of 10-fold higher (>) or lower (<) titer by PRNT used as cut-off values.

*Modified from Rabe IB, Staples JE, Villanueva J, et al. Interim Guidance for Interpretation of Zika Virus Antibody Test Results. *MMWR* 2016;65. DOI: <http://dx.doi.org/10.15585/mmwr.mm6521e1>.

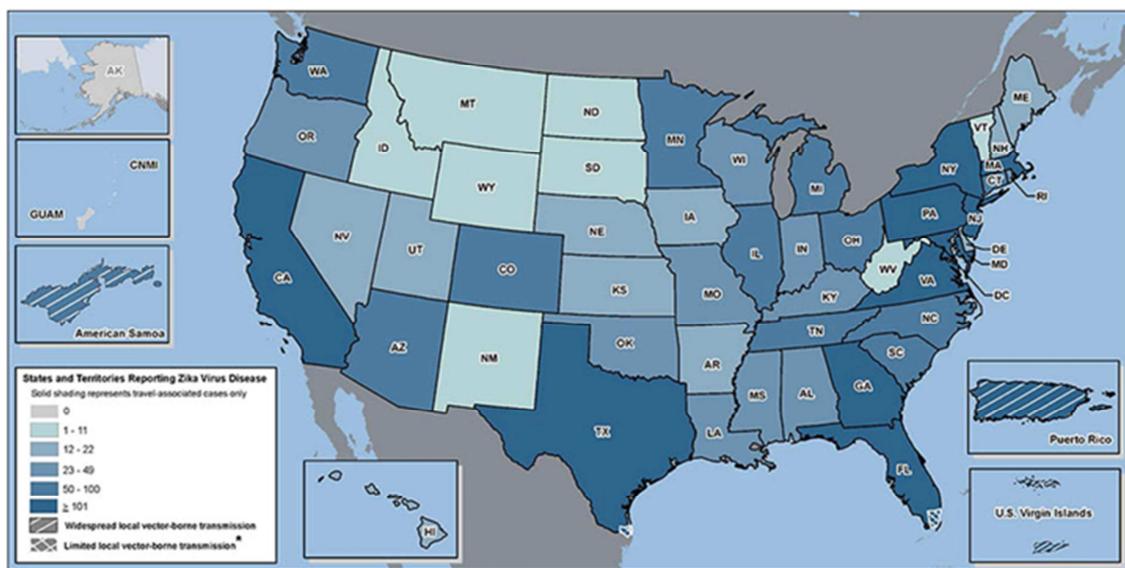
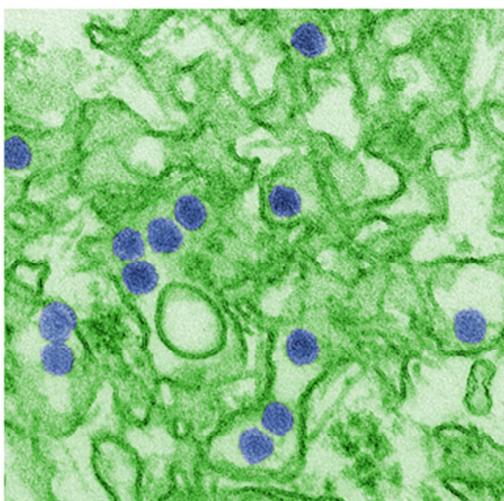
Table 3: CNS abnormalities reported with ZIKV during pregnancy

Ultrasound View	Abnormality	Specificity for Congenital ZIKV Infection
Transventricular (axial view)	<ul style="list-style-type: none"> Ventriculomegaly Septations in occipital horns 	<ul style="list-style-type: none"> Poor Poor
Transthalamic (axial view)	<ul style="list-style-type: none"> Agenesis of the thalamus 	<ul style="list-style-type: none"> Poor
Transcerebellar (coronal and axial views)	<ul style="list-style-type: none"> Posterior fossa abnormalities, including Vermian dysgenesis/hypoplasia and an enlarged cisterna magna 	<ul style="list-style-type: none"> In isolation, poor
Midsagittal plane (sagittal view)	<ul style="list-style-type: none"> Brain stem hypoplasia and/or atrophy Agenesis/Dysgenesis of the corpus callosum 	<ul style="list-style-type: none"> Poor Poor
Transcaudate (coronal view)	<ul style="list-style-type: none"> Enlargement of anterior horns of ventricular system Enlarged subarachnoid space that can be quantified by measuring sinocortical and craniocortical spaces. 	<ul style="list-style-type: none"> Poor
Brain parenchyma and cortex (can be assessed in all views)	<ul style="list-style-type: none"> Calcifications, predominantly located in the gray-white matter junction, but also identified in thalamus, basal ganglia, cortex and periventricular regions Brain atrophy with enlarged extra-axial spaces Abnormalities in the cortical development such as delayed sulcation, lissencephaly, polymicrogyria or pachygryia Enlarged confluence of the dural venous sinuses from intracranial hemorrhage 	<ul style="list-style-type: none"> Increased specificity for congenital viral and pathogen malformations, but not unique to congenital ZIKV Poor Poor Likely higher specificity for congenital ZIKV infection, but more data needed
Head profile	<ul style="list-style-type: none"> Slanted forehead, consistent with microcephaly 	<ul style="list-style-type: none"> Increased specificity for congenital viral and pathogen malformations, but not unique to congenital ZIKV
Orbits	<ul style="list-style-type: none"> Ocular defects (asymmetrical microphthalmia, cataracts and herniation of the orbital fat into the cranial vault) 	<ul style="list-style-type: none"> Increased specificity for congenital viral and pathogen malformations, but not unique to congenital ZIKV
Amniotic fluid assessment	<ul style="list-style-type: none"> Oligohydramnios 	<ul style="list-style-type: none"> Poor
Biometry: • BPD and HC • AC • FL, HL	<ul style="list-style-type: none"> Microcephaly/diminished head size Asymmetric growth restriction Symmetric growth restriction, or constitutional 	<ul style="list-style-type: none"> Poor, and may be genomic in origin Poor specificity in isolation
Extremities	<ul style="list-style-type: none"> Joint contractures (arthrogryposis) and clubbed feet 	<ul style="list-style-type: none"> Poor, and may be disruptive (<i>i.e.</i>, Potter's sequence), genomic or infectious in etiology

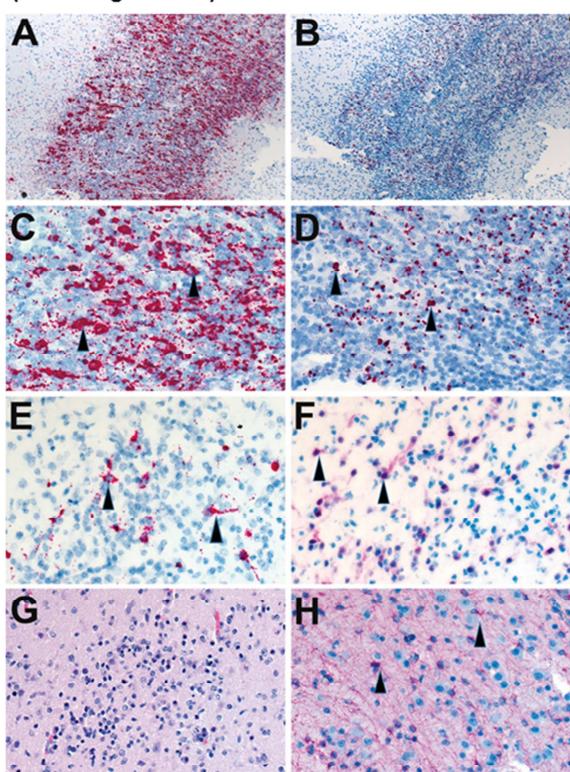
BPD: Biparietal diameter; HC: head circumference; AC: abdominal circumference; FL: femur length; HL: humerus length

A. Zika Cases Reported in the United States

Laboratory-confirmed Zika virus disease cases reported to ArboNET by state or territory (as of December 14, 2016)

**B.****C.**

ZIKV in Fetal Brain Tissue
(26 week gestation)



ZIKV in Placental Tissue
(spontaneous 1st trimester losses)

