On a quest for the eureka moment in Zika virus testing

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June 2016—The Zika virus testing challenges facing laboratories are many, including the virus' low and short-term viremia and its resemblance to other flaviviruses, especially dengue and chikungunya. "These viruses present extremely similarly, and it's very difficult to tell them apart based on clinical characteristics alone," said Benjamin Pinsky, MD, PhD, who presented the details of diagnostic tests for Zika in an Association for Molecular Pathology webinar in April.

To diagnose Zika infection in its acute phase, the CDC recommends that molecular testing for Zika virus RNA be performed first—within seven days of symptom onset—because of the extent of cross-reactivity in flavivirus serological assays, said Dr. Pinsky, an assistant professor of pathology and medicine in the Division of Infectious Diseases and Geographic Medicine at Stanford University School of Medicine.

The recommendation is to perform real-time PCR for Zika virus and for dengue and chikungunya.

Most of the nucleic acid amplification testing is performed on serum, he noted, and, in the literature, the assay performed most frequently is the CDC Zika real-time reverse-transcriptase PCR. It is based on sequencing derived from a 2007 outbreak in the Yap Islands in the Federated States of Micronesia.

The CDC ZIKV rRT-PCR assay consists of two separate one-step reactions targeting the premembrane and envelope genes. To be considered positive, both reactions must produce cycle threshold values of less than 38.5.

Of 157 serum samples collected during the Yap Islands outbreak, 17 tested positive, with 88.2 percent of the positives collected during the first three days of illness, suggesting patients are not viremic for long. A short duration of viremia was seen also in a later outbreak in French Polynesia.

The positive samples in the Yap Islands study had a mean Zika virus level of 4.4 log10 copies/mL of serum. "This is relatively low for these arboviral infections," said Dr. Pinsky, who is medical director of Stanford's clinical virology laboratory and a member of the CAP's Microbiology Resource Committee.

Testing this year in Nicaragua using a multiplex assay developed at Stanford (more on that later) found the mean Zika virus level to be similar to that seen in the Yap Islands outbreak: 4.9 log10 copies/mL of serum (with a standard deviation of one). In addition, the Zika virus level was found to be statistically significantly lower than the mean level for dengue and chikungunya, Dr. Pinsky said.

In the outbreak in French Polynesia in 2013–14, the CDC assay was performed on saliva and on serum samples. Both serum and saliva yielded similar results for the mean day of illness (3.3 versus 3.5, respectively). But "significantly more of the patients were positive in saliva than in serum, and this was statistically significant," Dr. Pinsky said. "So saliva may be a nice alternative specimen type to detect more cases of Zika infection." Because many of the patients were both serum- and saliva-positive, it might make sense to use both specimen types in testing for Zika virus, he suggests.

During a New Caledonia outbreak in 2014, urine was used as a specimen in addition to serum. Researchers found that Zika may be detectable in urine for seven or more days after it is no longer detectable in serum, Dr. Pinsky noted. However, a pregnancy study in Brazil in 2015 found that Zika RNA was more frequently detected in serum compared with urine: 13.6 percent (12/88) were urine positive and serum negative versus 29.5 percent (26/88) who were urine negative and serum positive.

"This suggests that getting both a serum and a urine would increase the detection of Zika in these patients," he said. "And perhaps getting serum, urine, and saliva would increase it even more."

Zika virus has been detected also in amniotic fluid, cerebrospinal fluid, semen, and fetal tissue.

Dr. Pinsky and Jesse Waggoner, MD, also of Stanford's Division of Infectious Diseases and Geographic Medicine, compiled a list (Waggoner JJ, Pinsky BA. J Clin Microbiol. 2016;54:860–867) of RT-PCR assays for detecting Zika virus, published in various journals between 2008 and this year.

Due to the short duration of Zika's viremia, the CDC recommends that symptomatic and high-risk patients who test negative for Zika virus in PCR assays undergo antibody testing, even though current immunoglobulin M assays don't reliably distinguish Zika from dengue and chikungunya.

In March, the FDA announced emergency use authorization for the CDC Trioplex Real-Time RT-PCR assay. It issued EUA for the Quest/Focus Zika Virus RNA Qualitative Real-Time RT-PCR in April (too late for Dr. Pinsky to share data). Other laboratories and diagnostics manufacturers have submitted data to the FDA. "I suspect we'll have many more options for Zika virus testing in the coming months," he said.

The CDC multiplex TaqMan assay can be used to detect and differentiate the RNA of Zika, dengue, and chikungunya in serum and cerebrospinal fluid and to detect Zika virus RNA in urine and amniotic fluid. "The assay showed excellent in silico inclusivity and exclusivity," Dr. Pinsky said. The CDC performed studies that showed no cross-reactivity with other flaviviruses, including West Nile, yellow fever, and St. Louis encephalitis viruses.

But this new CDC assay comes with disclaimers, Dr. Pinsky noted. First is the lower limit of detection, which he said is relatively high: 4.19 log10 genome copy equivalent per mL of serum for Zika (and 4.13 log10 for dengue-4 and 5.60 log10 for chikungunya). "The lower limit of detection is close to the mean value in patients," he said. Co-infections were not evaluated, he added, and the data indicate they may be common.

Second, it was not extensively tested with clinical specimens. "This is really a weakness of many of the assays," he said. "Evaluations of specimens in the countries that are having ongoing transmission have not yet been performed."

The approval of the CDC Trioplex rRT-PCR was obtained with just two urine and two amniotic fluid samples. And it was compared with Zika and chikungunya assays of unknown performance characteristics and with a dengue assay that has been shown numerous times in the literature to be less sensitive than other molecular dengue assays.

"Importantly for laboratories throughout the world, the genomic targets were not disclosed," Dr. Pinsky said. "And, of course, testing is limited to qualified laboratories designated by the CDC."

Dr. Pinsky's team at Stanford developed its own ZCD rRT-PCR assay, which targets the NS4B gene. Combined in multiplex with chikungunya and dengue virus assays, this test uses TaqMan chemistry and its performance has been validated on multiple real-time PCR instruments.

He and colleagues designed the assay, the first of which was run in April 2014, using all complete or nearly complete Zika virus genome sequences available in GenBank as of March that year. As more sequences have come out, he said, they have confirmed that the primers and probes have a 100 percent match to strains from the Americas.

In the analytical evaluation, they found the linear range was broad and the lower limit of detection was very low, "at least 10-fold lower than what's described for the Trioplex assay," Dr. Pinsky said. They examined exclusivity by testing genomic RNA from a large number of related viruses, and no amplification was observed.

The clinical evaluation was performed this year in Nicaragua. "Zika virus was detected in significantly more Nicaraguan samples using our ZCD assay than the comparator," Dr. Pinsky said. Interestingly, about 30 percent of the positives showed evidence of mixed infection.

Before it authorized emergency use of the Trioplex assay, the FDA in February issued emergency use authorization for the CDC Zika IgM antibody capture enzyme-linked immunosorbent assay (Zika MAC-ELISA).

“Zika IgM can be detected about four days after the onset of illness and may persist for 12 weeks or longer,” Dr. Pinsky said. “Previous infection or vaccination with other flaviviruses like dengue, Japanese encephalitis, or yellow fever may result in false-positive IgM results.”

The CDC’s serological testing algorithm says patients who test positive for Zika virus with an IgM assay need to undergo a plaque reduction neutralization test, or PRNT. “The positive percent agreement is good between the IgM assay and PRNT,” Dr. Pinsky said, referring to the 97.8 percent (45/46) agreement reported in the IgM ELISA package insert. “However, the negative percent agreement, or the specificity, is pretty poor,” at 45.5 percent (45/99).

In addition to the low specificity, there are other disclaimers. PRNT is unable to resolve 17.2 percent (16/93) of Zika MAC-ELISA positive specimens. MAC-ELISA and PRNT testing are limited to qualified laboratories designated by the CDC. And PRNT is laborious, Dr. Pinsky said. “It takes some time for the results to be returned to clinicians.”

For pregnant women, the CDC recommendation is to perform antibody testing and, if positive, to perform dengue IgM testing because of the possibility of cross-reactivity. If it’s negative, it’s a probable Zika virus. No recommendations are provided beyond that, but amniotic fluid testing can be considered if indicated, Dr. Pinsky said. If the patient is positive for dengue, it’s an unspecified flavivirus infection and PRNT testing may be helpful in discriminating between Zika and other flaviviruses.

For neonates, serum and CSF can be tested for both RNA and antibodies. The placenta and umbilical cord can be evaluated histopathologically, by immunohistochemistry staining on fixed tissue, and by RT-PCR on fixed and frozen tissue. Maternal serum can be tested if it has not been done.

Dr. Pinsky is hopeful that NAAT and antibody tests will soon become more widely available and that better assays will continue to be developed and testing turnaround times reduced. “Folks are working on the identification of Zika virus-specific epitopes, and then the reverse of the leading common epitopes, so hopefully that will improve Zika virus IgM testing,” he said. “And I think it’s important to develop alternatives to plaque reduction neutralization testing,” such as Zika virus Western blot, which he says is being worked on at the University of Washington. Then, too, there is the development of specific Zika virus NS1 assays, the latter of which are commonly used for dengue and could be helpful to rule out Zika in locations where molecular testing cannot be performed. And it’s important, he said, to move all of these assays to near-care and point-of-care testing.
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