Development of flow cytometry-based Zika virus detection assay

Sojan Abraham, Steven Wood

Division of Biology, Chemistry, and Materials Science, Office of Science and Engineering Laboratories, US FDA-Center for Devices and Radiological Health, 10903 New Hampshire Ave, Silver Spring, Maryland, USA, 20993

Summary. – Standard assays based on ELISA and RT-PCR have been widely used to detect flaviviral infections, including the Zika virus. Despite their simple, unique, and sensitive features, RT-PCR and ELISA-based assays cannot meet the requirements of high-throughput screening of bulk samples during an outbreak. Several research groups around the world are working on the development of rapid, multiplex, and sensitive assays to overcome the limitations of standard assays used in viral detection. Recent advances in flow cytometry have led to remarkable progress in its use as a basic analysis tool in laboratories. Here, we used the advantages of flow cytometry to develop a Zika virus detection assay using recombinant Zika virus envelope (E) protein. The E protein-based flow cytometry assay was able to detect anti-Zika E antibodies from Zika-infected patients, Zika-infected mice, and mice immunized with recombinant Zika E protein. We report the development of the first flow cytometry-based diagnostic assay that can be used for Zika detection. Its rapid turnaround time and ability to detect antibodies from Zika-infected patients can be used to improve the diagnostic accuracy of Zika virus detection.

Keywords: Flavivirus; Zika virus; E protein; NS-1 protein; flow cytometry; ELISA; RT-PCR

Introduction

Flaviviruses belong to the family *Flaviviridae,* which includes several human pathogens such as Zika virus, West Nile virus (WNV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), and Dengue virus (DENV). Zika is an arthropod-borne virus, first identified in 1947 from an ill rhesus monkey in Uganda's Zika forest. The first confirmed case of human Zika virus infection was reported in Uganda in 1962–1963 (Dick *et al.*, 1952; Wikan and Smith, 2016). It is known to cause mild febrile illness and can occasionally lead to potentially life-threatening outcomes such as microcephaly, congenital malformations, and Guillain-Barre syndrome (Weaver *et al.*, 2016).

Standard assays based on ELISA and RT-PCR have been extensively used to detect Zika virus infections (Announcement: Guidance for US, 2016; Faye *et al.*, 2008). One major obstacle in developing serological assays that distinguish Zika virus infections from those caused by other flaviviruses is the presence of cross-reactive antibodies. The E protein-based IgM capture (MAC) ELISA developed by InBios International has been authorized by the Food and Drug Administration (FDA) under an emergency use authorization (EUA). However, due to cross-reactivity with other flaviviruses, results may be difficult to interpret and require confirmation by plaque reduction neutralization (PRNT) assays to eliminate the false positive cases. Non-structural (NS) protein-based serological assays have been developed either alone or in combination with E protein. Two described non-structural protein 1 (NS-1) based serological assays are the IgM capture ELISA from NovaTec and the indirect ELISA from EuroImmun (Basile *et al.*, 2018). Zika virus detection assays utilizing Luminex and nanotechnology platforms are also reported in the literature. These assays employing Zika non-structural proteins utilize measurements of different antibody subtypes to improve

Corresponding authors. E-mails: Sojan.abraham@fda.hhs.gov, Steven.wood@fda.hhs.gov; phone: +1-301-796-7971.
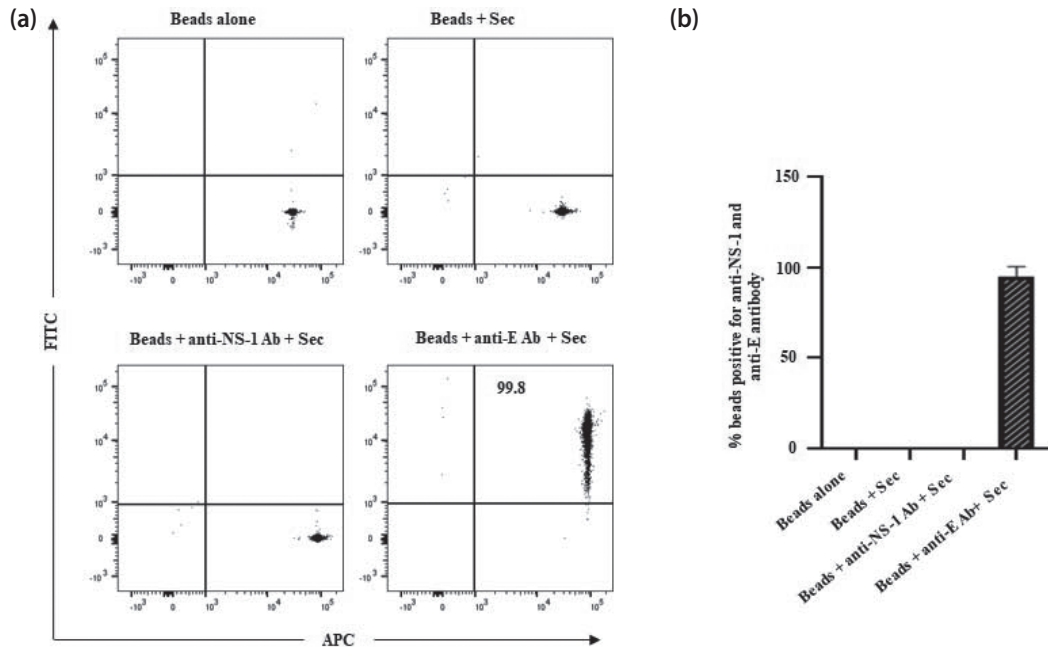
Fig. 1

Testing the specificity of E protein conjugation

Zika E conjugated beads were incubated with anti-Zika NS-1 and E antibodies. Primary antibody-stained beads were detected by staining with a FITC conjugated secondary antibody. Stained beads were later analyzed using a BD FACSCanto™ II Flow Cytometer. **(a)** representative data from one of the four independent experiments, and **(b)** cumulative data from four independent experiments.

the specificity of Zika virus detection (Wong *et al.*, 2017; Zhang *et al.*, 2017).

RT-PCR-based Zika virus detection assays are widely used in Zika diagnosis, but the narrow temporal window in which viral RNA is present in sera and urine limits their use (Calvet *et al.*, 2016; Lanciotti *et al.*, 2008). Despite their simple, unique, and sensitive features, ELISA and RT-PCR-based viral detection assays cannot meet the requirements of high-throughput screening and are not suitable for the urgent screening of bulk samples during a viral outbreak. Therefore, developing a rapid, high-throughput, and accurate screening assay is essential for detecting and differentiating flaviviral infections. A viral detection assay using a flow cytometry platform has several advantages over conventional ELISA assays. These include rapid turnaround time and the ability to detect multiple parameters (e.g., antibody isotypes) simultaneously. Therefore, the objective of this work was to develop a high-throughput Zika virus detection assay in a flow cytometry platform using Zika E protein.

Materials and Methods

LEGENDplex carboxyl beads containing allophycocyanin (APC) fluorescence (BioLegend, USA) were used for the assay development. The surface chemistry of these beads allows for the covalent coupling of different antigens. Antigen conjugation to these beads can be detected using a secondary antibody coupled to a fluorochrome other than APC. The bead conjugation was carried out according to the manufacturer's recommendations.

Briefly, LEGENDplex carboxyl beads were washed and resuspended in coupling buffer, then activated by incubation with N-hydroxysulfosuccinimide (Thermo Fisher Scientific, USA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (Thermo Fisher Scientific) for 20 min at room temperature. After activation, the beads were rewashed and resuspended in a coupling buffer. Zika E proteins were later conjugated to activated carboxyl beads by vortexing and incubation at room temperature for 2 h. Conjugated beads were then blocked with blocking buffer and used for flow cytometric assays. The protein conjugation and the specificity of these beads were confirmed by staining with anti-E (BEI Resources, USA) and anti-NS-1 (Thermo Fisher Scientific) antibodies. Protein conjugated beads were first stained with primary antibodies for 45 min at room temperature and then washed with staining buffer (PBS with 2%FBS) followed by incubation with FITC (fluorescein isothiocyanate) conjugated secondary antibody (Jackson Immuno Research Labs, USA) for 45 min at room temperature. They were then rewashed and analyzed using a BD FACSCanto™ II flow cytometer.

Results

As shown in Fig. 1a,b, more than 99% of E protein conjugated beads were positive for E and negative for NS-1 specific antibodies, confirming the protein conjugation and specificity of these beads. The E protein conjugated beads were then used to detect anti-Zika E antibodies in human sera collected from Zika infected donors. This study was conducted under the approved IRB protocol 17346-162945458z "Human immune responses to Zika." Zika positive and negative human serum samples were purchased from Boca Biolistics (Pompano Beach, USA). The company confirmed Zika virus infection status of these individuals using ZIKV Detect™ IgM capture ELISA kit from InBios (data not shown). Zika E-protein conjugated beads were incubated with different dilutions (1:1,000, 1:5,000, and 1:25,000) of human sera collected from Zika-infected and uninfected donors to identify the optimum serum dilution for the assay. After standardization, 1:5,000 dilution was selected for the assay, and this high dilution of serum was necessary to reduce the background fluorescence. After incubating with diluted serum for 45 min at 4°C, the beads were washed once

with wash buffer, followed by incubation with biotin-conjugated goat anti-human IgG/A/M antibody (Thermo Fisher Scientific) for 45 min at 4°C. Next, the beads were washed once with wash buffer and stained with streptavidin-PE conjugated detection antibody (Jackson Immuno Research Labs) for 45 min at 4°C. Next, the beads were washed once with wash buffer and analyzed using a BD FACSCanto™ II flow cytometer. As shown in Fig. 2a, b, all 20 presumptive Zika positive serum samples tested were positive for anti-Zika E antibody, confirming these donors' previous Zika virus infection status. Of 14 presumptive Zika-negative samples tested, 13 were negative for anti-Zika E antibodies; we found anti-E antibodies in one sample. Our results demonstrated that E conjugated beads could detect anti-Zika E antibodies in Zika infected patients.

To further demonstrate the usefulness of flow cytometry-based assays to detect anti-Zika E antibodies, we studied the humoral immune response in mice following their immunization with Zika E and NS-1 proteins. C57/BL6 mice were obtained from Jackson Laboratory (Bar Harbor, USA) and maintained according to guidelines outlined in the National Institutes of Health manual *Guide*
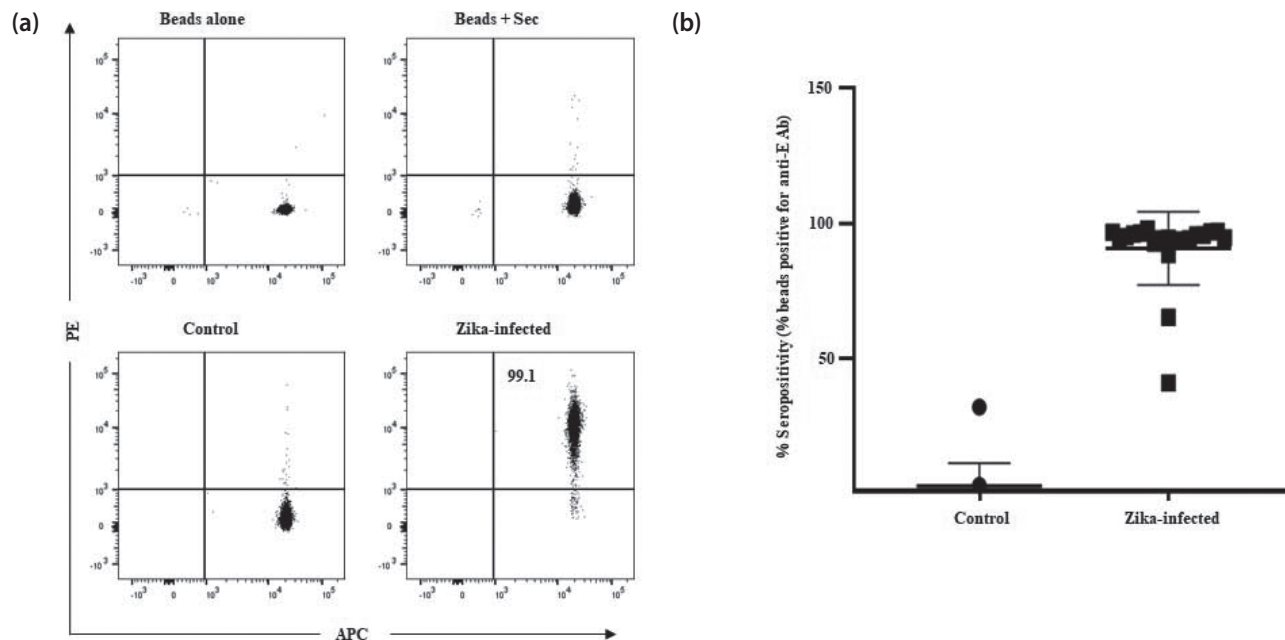

Fig. 2

Flow cytometric analysis of serum samples collected from Zika-infected and uninfected donors using E protein conjugated beads
Zika E protein conjugated beads were incubated with serum collected from Zika-infected and uninfected donors. The beads were stained with biotin-conjugated goat anti-human IgG/A/M antibody and streptavidin-PE conjugated detection antibody. Antibody-stained beads were subsequently analyzed using a BD FACSCanto™ II flow cytometer. **(a)** Representative flow cytometric data from one of the three independent experiments. Unstained E conjugated beads (Beads alone), beads with biotin-conjugated secondary antibody (Beads + Sec), beads with serum samples collected from uninfected donors, (control), and beads with serum samples collected from Zika-infected donors, (Zika-infected). **(b)** Cumulative data from sera collected from 14 uninfected (control) and 20 Zika-infected donors. Each symbol represents the mean of triplicates from each donor.

for the Care and Use of Laboratory Animals. The FDA's White Oak Institutional Animal Care and Use Committee approved the animal study protocol, and the study was performed under protocol number 2017-56. C57/BL6 mice were immunized with 2.0 μg of Zika E protein plus adjuvant (montanide iso 206 VG). The animals were immunized twice at a 10-day interval. These animals were euthanized on day 20, and serum was collected and used for flow cytometric analysis. As a control, we immunized the mice with recombinant Zika NS-1 protein (RayBiotech, USA). The blood was isolated following a cardiac puncture, and serum was collected and used for flow cytometric analysis. E and NS-1 conjugated beads were incubated with different dilutions of the serum (1:1,000 and 1:10,000

dilution) to identify the optimum serum dilution for the assay. After standardization, 1:10,000 dilution of serum was used for the experiment. Here again, the high dilution of serum was essential for reducing the background fluorescence. The beads were washed once with wash buffer 45 min after incubation, then stained with biotin-conjugated goat anti-mice IgG secondary antibody for 45 min at 4°C. They were then rewashed with wash buffer and stained with streptavidin PE-conjugated detection antibody (Jackson Immuno Research Labs) for 45 min at 4°C. Finally, the beads were washed with wash buffer and analyzed using a BD FACSCanto™ II flow cytometer. As shown in Fig. 3a, anti-Zika E antibodies were detected in mice immunized with E protein. In contrast, no anti-Zika
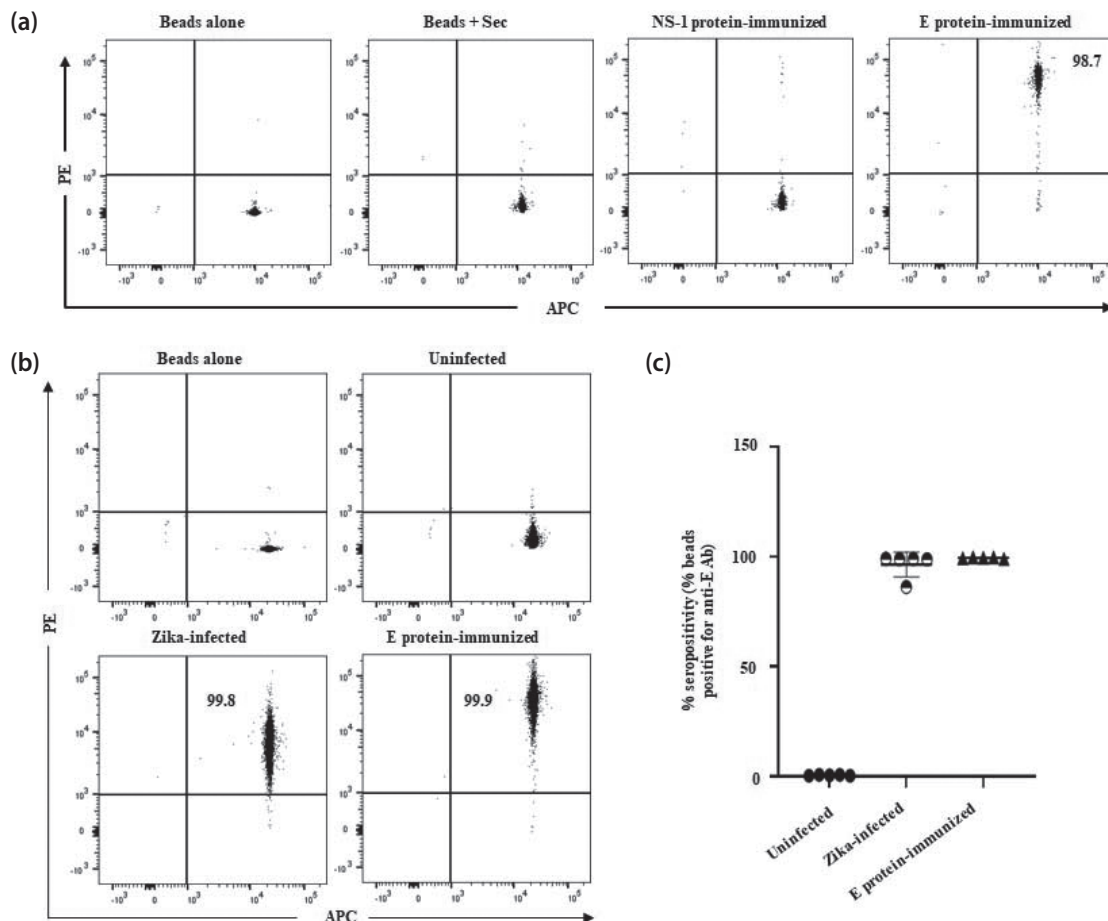

Fig. 3

Flow cytometric analysis of serum collected from Zika-infected, NS-1, and E protein-immunized mice

(a) Zika E protein conjugated beads were incubated with serum (1:10,000 dilution) collected from NS-1 and E protein-immunized animals (n = 3). **(b)** and **(c)** flow cytometric analysis of serum collected from uninfected, Zika-infected, and E protein-immunized mice. Zika E protein conjugated beads were incubated with serum collected from uninfected, Zika-infected, and E protein-immunized mice (n = 5). **(b)** Representative flow cytometric data from one of the three independent experiments. Unstained E protein conjugated beads (Beads alone), beads with serum samples collected from uninfected mice (Uninfected), beads with serum samples collected from Zika-infected mice (Zika-infected), and beads with serum samples collected from E protein-immunized mice, (E protein-immunized) are shown. **(c)** Cumulative data from sera collected from uninfected, Zika-infected, and E protein-immunized mice. Each symbol represents the mean of triplicates from each individual mouse.

E antibodies were detected in NS-1 protein-immunized animals.

To further demonstrate the consistency and reproducibility of this assay, the E protein conjugated beads were used for detecting E protein-specific antibodies present in Zika infected mice. Recent studies demonstrated that administration of anti-IFNAR monoclonal antibodies in C57/BL6 WT mice one day before Zika inoculation leads to Zika-induced disease, neuroinflammation, and mortality (Smith *et al.*, 2017). Zika strain DAK AR 41524 was obtained from BEI Resources (Manassas, USA). The virus was titrated and amplified using VERO cells (ATCC, CCL-81). Eight-week-old C57/BL6 mice (n = 5/group; Jackson Laboratories) were intra peritoneally injected with a total of 3.0 mg (2.0 mg first dose, 0.5 mg subsequent doses) of MAb-5A3 (Leinco Technologies, USA) or PBS on day -1, day +1, and day 4. On day 0, mice were infected with the Zika strain DAK AR 41524 by an intra peritoneal exposure route in a total volume of 200 μl. The serum was collected on day five and used for flow cytometric analysis. E protein-immunized animals were used as positive controls. As shown in Fig. 3b,c, anti-Zika E antibodies were detected in mice infected with Zika and E protein-immunized mice, whereas no anti-Zika E antibodies were detected in uninfected mice. The data further confirm that Zika E protein-based flow cytometry assays can be used to detect Zika E-specific antibodies.

Discussion

A viral detection assay using a flow cytometry platform has several advantages over the well-established ELISA platform. These include rapid turnaround time and the ability to simultaneously detect multiple parameters (e.g., antibody isotypes). Our results reported herein clearly demonstrate the usefulness of this assay and describe the development of the first flow cytometry-based antibody detection assay to evaluate Zika virus infection. In addition, data showed that the assay is quick (around 3 h for completion), requires fewer sample volumes (1:5,000 diluted serum samples), and is more cost-effective than a commercial ELISA assay.

One major obstacle in developing specific serological assays that distinguish Zika virus infection from those caused by other flaviviruses is the presence of cross-reactive antibodies in infected individuals. Our initial results demonstrated that the flow- cytometry-based assay has the potential to develop into a high-throughput Zika diagnostic assay. However, E protein-based assays do not differentiate Zika virus infection from those caused by other flaviviruses because of their high sequence similarity and ability to induce cross-reactive antibodies against other flaviviruses. To overcome this challenge, work is currently underway to develop a flow-cytometry-based Zika diagnostic assay using the NS-1 protein as the reference material. This is based on a few previous studies which suggest that antibodies against flavivirus non-structural proteins may be virus type-specific (Garcia *et al.*, 1997; Shu *et al.*, 2002; Wong *et al.*, 2003). Another way to improve Zika diagnosis is to identify Zika specific peptide sequences within E and NS-1 proteins and use them as the reference materials in the Zika diagnostics assay. Work to identify these Zika specific epitopes is ongoing.

In conclusion, this study is the first to report the development of a flow- cytometry-based Zika virus detection assay. Its rapid turnaround time and ability to detect antibodies from Zika infected patients can be used to improve the diagnostic accuracy of Zika virus detection.

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References

(2016): Announcement: Guidance for U.S. Laboratory Testing for Zika Virus Infection: Implications for Health Care Providers. MMWR Morb. Mortal. Wkly. Rep. 65, 1304. https://doi.org/10.15585/mmwr.mm6546a7

Basile AJ, Goodman C, Horiuchi K, Sloan A, Johnson BW, Kosoy O, Laven J, Panella AJ, Sheets I, Medina F, Mendoza EJ, Epperson M, Maniatis P, Semenova V, Steward-Clark E, Wong E, Biggerstaff BJ, Lanciotti R, Drebot M, Safronetz D, Schiffer J (2018): Multi-laboratory comparison of three commercially available Zika IgM enzyme-linked immunosorbent assays. J Virol. Methods 260, 26–33. https://doi.org/10.1016/j.jviromet.2018.06.018

Calvet GA, Santos FB, Sequeira PC (2016): Zika virus infection: epidemiology, clinical manifestations and diagnosis. Curr. Opin. Infect. Dis. 29, 459–466. https://doi.org/10.1097/QCO.0000000000000301

Dick GW, Kitchen SF, Haddow AJ (1952): Zika virus. I. Isolations and serological specificity. Trans. R. Soc. Trop. Med. Hyg. 46, 509–520. https://doi.org/10.1016/0035-9203(52)90042-4

Faye O, Faye O, Dupressoir A, Weidmann M, Ndiaye M, Alpha Sall A (2008): One-step RT-PCR for detection of Zika virus. J. Clin. Virol. 43, 96–101. https://doi.org/10.1016/j.jcv.2008.05.005

Garcia G, Vaughn DW, Del Angel RM (1997): Recognition of synthetic oligopeptides from nonstructural proteins NS1 and NS3 of dengue-4 virus by sera from dengue virus-infected children. Am. J. Trop. Med. Hyg. 56, 466–470. https://doi.org/10.4269/ajtmh.1997.56.466

Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, Stanfield SM, Duffy MR (2008): Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg. Infect. Dis. 14, 1232–1239. https://doi.org/10.3201/eid1408.080287

Shu PY, Chen LK, Chang SF, Yueh YY, Chow L, Chien LJ, Chin C, Yang HH, Lin TH, Huang JH (2002): Potential application of nonstructural protein NS1 serotype-specific immunoglobulin G enzyme-linked immunosorbent assay in the seroepidemiologic study of dengue virus infection: correlation of results with those of the plaque reduction neutralization test. J. Clin. Microbiol. 40, 1840–1844. https://doi.org/10.1128/JCM.40.5.1840-1844.2002

Smith DR, Hollidge B, Daye S, Zeng X, Blancett C, Kuszpit K, Bocan T, Koehler JW, Coyne S, Minogue T, Kenny T, Chi X, Yim S, Miller L, Schmaljohn C, Bavari S, Golden JW (2017): Neuropathogenesis of Zika virus in a highly susceptible immunocompetent mouse model after antibody blockade of type I interferon. PLoS Negl. Trop. Dis. 11, e0005296. https://doi.org/10.1371/journal.pntd.0005296

Weaver SC, Costa F, Garcia-Blanco MA, Ko AI, Ribeiro GS, Saade G, Shi PY, Vasilakis N (2016): Zika virus: History, emergence, biology, and prospects for control. Antiviral. Res. 130, 69–80. https://doi.org/10.1016/j.antiviral.2016.03.010

Wikan N, Smith DR (2016): Zika virus: history of a newly emerging arbovirus. Lancet Infect. Dis. 16, e119-e126. https://doi.org/10.1016/S1473-3099(16)30010-X

Wong SJ, Boyle RH, Demarest VL, Woodmansee AN, Kramer LD, Li H, Drebot M, Koski RA, Fikrig E, Martin DA, Shi PY (2003): Immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections and from flavivirus vaccination. J. Clin. Microbiol. 41, 4217–4223. https://doi.org/10.1128/JCM.41.9.4217-4223.2003

Wong SJ, Furuya A, Zou J, Xie X, Dupuis AP 2nd, Kramer LD, Shi PY (2017): A Multiplex microsphere immunoassay for Zika virus diagnosis. EBioMedicine 16, 136–140. https://doi.org/10.1016/j.ebiom.2017.01.008

Zhang B, Pinsky BA, Ananta JS, Zhao S, Arulkumar S, Wan H, Sahoo MK, Abeynayake J, Waggoner JJ, Hopes C, Tang M, Dai H (2017): Diagnosis of Zika virus infection on a nanotechnology platform. Nat. Med. 23, 548–550. https://doi.org/10.1038/nm.4302