Highly Sensitive and Selective Direct Detection of Zika Virus Particles in Human Bodily Fluids for Accurate Early Diagnosis of Infection

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ABSTRACT: Zika virus (ZIKV) is an arbovirus that caused widespread panic beginning in 2015 in northeastern Brazil due to the threatening link between infection and fetal abnormalities such as microcephaly, spontaneous abortions, and stillbirths. Since the epidemic began, the virus has been further investigated, unveiling that the long-term dangers of ZIKV infection go beyond fetal neurological impairment. Characterization of the active infection has proven difficult as only 20% of infected individuals are symptomatic. Additionally, ZIKV is often misdiagnosed due to serological cross-reactivity with similar flaviviruses such as dengue, yellow fever, and West Nile. To date, there is no approved vaccine or therapy against ZIKV, highlighting the urgent need to accurately identify active infection to help minimize the spread of the virus. Herein, we describe a highly specific and sensitive enzyme-linked immunosorbent assay to detect early active ZIKV using neutralizing human monoclonal antibodies isolated from infected patients in Brazil that do not cross-react with dengue viruses 1–4 and bind directly to a ZIKV immunodominant epitope. The calculated limits of detection of active ZIKV fall within the physiological ranges of the virus in human bodily fluids. This selective immunoassay creates the platform required for future translation toward a point-of-care assay for ZIKV, a necessity to diagnose active ZIKV in the remote regions of which it thrives.

INTRODUCTION

Zika virus (ZIKV) is an enveloped single-stranded ribonucleic acid (RNA) flavivirus that was first isolated over 70 years ago from rhesus monkeys in the Zika Forest of Uganda. A detailed investigation was not initially performed on ZIKV, being that an estimated 80% of cases of infection are asymptomatic, and those individuals that do present symptoms are often misdiagnosed as having similar febrile illnesses such as dengue virus (DENV). In early 2015, there was a rapid and widespread infection of ZIKV in Brazil, which was identified due to an over 28-fold increase in infants born with microcephaly in the region. This epidemic resulted in a further investigation of the dangers of ZIKV infection such as severe neurological impairments of the infants born to infected mothers and Guillain–Barre syndrome in infected adults among other health complications. The various routes of transmission of the virus, including spread via Aedes mosquito species, sexual transmission, and perinatal and blood transfusions, resulted in ZIKV penetrating the Americas, leading the World Health Organization to declare ZIKV a Public Health Emergency of International Concern (PHEIC) on February 1, 2016. Since then, there has been a large increase in the efforts focused on the characterization of the virus and its corresponding detection, as symptomatic cases closely resemble DENV, a mosquito borne viral infection demonstrating similar initial generic symptoms such as muscle and joint pain, fever, rash, and conjunctivitis. Ongoing research is also being conducted in the area of vaccine development since there is no current therapy or vaccine against ZIKV.

The development of sensitive and selective methods for the detection of ZIKV is a relatively new field, and because of this, there is no gold standard for the detection of ZIKV. Accordingly, there are various assays that have been developed recently that utilize technologies such as reverse transcription polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), nucleic acid sequence-based amplification, chemiluminescence-based immunoassays, neutralizing assays, and enzyme-linked immunosorbent assays (ELISAs). The nucleic acid-based assays detect the presence of ZIKV RNA. While these recently
developed molecular-based tests are sensitive and selective, sample preparation of some sort is traditionally required to extract the viral RNA from the sample matrix for its corresponding identification. Often times, a purification step is also employed following viral lysis. Similar to nucleic acid-based assays, antibody-based assays detect the presence of ZIKV in an indirect manner by detecting the antibodies generated against ZIKV. Antibody-based assays specifically detect immunoglobulin G (IgG) and immunoglobulin M (IgM), the antibodies produced by the body in response to viral infection, useful tools for the identification of previous viral infection.1 In contrast to these indirect detection methods, electrochemiluminescence-based immunoassays detect active ZIKV.9,10 Unfortunately, these systems do not easily lend for further translation to a point-of-care device due to the aqueous restrictions needed for the electrodes.11 Lastly, apart from these currently available assays, plaque reduction neutralizing tests (PRNTs) are often performed for ZIKV identification. The PRNT assays provide the most accurate means of detection for ZIKV; however, its protocol requires several days for obtaining results and, thus, is not practical and amenable for incorporation into point-of-care platforms. Therefore, there is a need to monitor active infection by employing other methods that can be translated into point-of-care detection.

Considering the drawbacks of the currently available detection methods, there is still a need for a method capable of detecting an early and active Zika virus infection. Accordingly, we have developed a highly selective and sensitive immunoassay for the direct detection of active ZIKV in human bodily fluids. Assay development was accomplished by harnessing the unique binding properties of three novel human-neutralizing monoclonal antibodies (mAbs) purified from infected patients in Brazil that do not cross-react with any of the four dengue virus serotypes and bind to ZIKV directly for the identification of ZIKV infection at the earliest time point possible.12 The implementation of these highly selective novel mAbs to an ELISA provides direct viral infection detection as compared to currently available immunoassays that detect the production of IgM and IgG antibodies in response to ZIKV infection.

Our ELISA-type test is currently used in a laboratory setting. However, our test is amenable for translation into a paper-based lateral flow point-of-care assay, which should be inexpensive, portable, and meet the WHO-assured requirements. In such tests, the most expensive reagents are usually the antibodies. Our test uses antibodies prepared from our own clones, which reduces the cost of their manufacturing. After corresponding calculations, we estimate that our test would be approximately $0.25, which is comparable in cost to other lateral flow point-of-care tests of which the average cost is $0.22.13

In contrast to the existing commercial assays that use indirect measures of past or present infection, the test reported herein diagnoses the active infection of ZIKV using three novel human neutralizing anti-ZIKV mAbs. The significance of developing such a test for active ZIKV infection is important when considering the dangerous risks associated with ZIKV infection of pregnant women, such as abnormal fetal development or other neurological effects to both the fetus and the mother. The availability of a test that can be performed during a physician’s visit would help in diagnosing potential ZIKV infection in a timely fashion, allowing for therapeutic intervention to be implemented immediately.14 Such a test could also be useful for couples who are planning a pregnancy and live in locations where Zika infection is prevalent.

### RESULTS AND DISCUSSION

There has been an ongoing need for a reliable, sensitive, simple, and cost-effective detection method for ZIKV. During the active viremia cycle, ZIKV can be detected in most human bodily fluids including urine, serum, whole blood, semen, breast milk, and saliva, two of which are easily and noninvasively collected from patients of both sexes: urine and saliva. While semen contains the highest detectable ZIKV levels for the longest period of time (Table 1), developing a test for semen would inherently exclude the female population. Likewise, developing a test for breast milk would exclude the male population. Therefore, we have focused mostly on human urine, serum, whole blood, and saliva as our possible sample matrices during the development of our test as to not exclude either of the sexes.

Current assays for the detection of ZIKV are either (1) molecular-based techniques or (2) immunoassays. While highly sensitive and selective, molecular-based techniques such as RT-PCR and RT-LAMP are designed to detect ZIKV RNA to indirectly detect the virus. These methods require either a sample preparation step or additional incorporation of a lysis agent to free the RNA from the viral capsid and often times necessitate a subsequent purification step. The alternative antibody-based platforms are rapid and simple but subject to the selectivity and binding characteristics of the antibodies themselves. The appeal of our work comes from a trio of highly selective antibodies against ZIKV. These antibodies were expressed and purified from Expi293F human cell lines, as described in the Methods section, which were cloned from the cDNA of three patients originally isolated from three volunteers from São Paulo, Brazil.

It is imperative to develop a test that accurately detects the desired virus particle in a patient sample with no cross-reactivity, ensuring that the test is specific for the desired

### Table 1. Zika Levels in Human Bodily Fluids

| bodily fluid | Zika levels (copies/mL) | detectable for | methods of detection | references |
|--------------|-------------------------|----------------|----------------------|------------|
| serum        | (8.1–30) × 10^6         | 1–11 days      | ELISA, RT-PCR, real-time RT-PCR, PRNT | 15–23      |
| saliva       | (0.02–90) × 10^6        | 1–8 (29 days)  | ELISA, RT-PCR, real-time RT-PCR, PRNT | 15, 16, 22–24 |
| urine        | (0.2–3000) × 10^4       | 2 to >20 (29 days) | ELISA, RT-PCR, real-time RT-PCR, PRNT | 15–20, 23, 25, 26 |
| semen        | (1.1–6.3) × 10^6        | 21–62 days     | real-time RT-PCR     | 23, 27–30 |
| breast milk  | (0.0004–2.1) × 10^6     | 3–8 days       | RT-PCR, real-time RT-PCR, RT-qPCR | 16, 23, 31 |

*ELISA: enzyme-linked immunosorbent assay; RT-PCR: reverse transcription polymerase chain reaction; real-time RT-PCR: real-time reverse transcription polymerase chain reaction; RT-qPCR: reverse transcription quantitative polymerase chain reaction; PRNT: plaque reduction neutralization test. *Reported in a single case.
antigen. To ensure that the developed immunoassay was specific to only ZIKV and not any of the four dengue virus serotypes (DENV1, DENV2, DENV3, DENV4) known to have homology in their sequence with the ZIKV and thus cross-react with antibodies against ZIKV, selectivity tests were carried out by running our immunoassay using either ZIKV, DENV1, DENV2, DENV3, or DENV4 spiked samples. Specifically, ELISA plates were coated with capture antibody, followed by incubating spiked samples containing ZIKV, or one of the DENV serotypes. After the virus samples are captured, our ZIKV-specific antibodies were added followed by a species-specific secondary antibody conjugated to horse-radish peroxidase (HRP). In the final step, a luminol-based chemiluminescent substrate was added, and the generated signal was measured using a luminescent microtiter plate reader. The sensitivity was calculated by first measuring the signal intensity for both ZIKV and DENV, using our assay, and comparing the percent signal intensity generated for each DENV serotype, at a viral concentration of $1.3 \times 10^8$ copies/mL. The signals obtained with the DENV 1–4 were compared with those obtained from ZIKV. The ratio between the DENV 1–4 signal and that of the ZIKV was found to be ranging between 8.2 and 29.2%, indicating the high selectivity of the antibody for ZIKV as compared to that for the DENV 1–4 serotypes (Figure 1).[32] These results demonstrate the highly selective nature of our immunoassay.

![Figure 1. Selectivity studies performed showing that clones P1F12, P1H09, and P1B04 bind selectively to ZIKV (calculated $p < 0.0001$). Statistical significance between ZIKV and DENV serotypes (1–4) was calculated using two-way analysis of variance (GraphPad Prism Ver. 8.4)].

After the identification of these selective antibodies, P1F12, P1H09, and P1B04, a sandwich-format colorimetric microtiter plate-based immunoassay was developed for the detection of ZIKV. The first step of assay optimization compared a luminol-based chemiluminescent substrate with 3,3′,5,5′-tetramethylbenzidine (TMB), an absorbance-based substrate. While the limits of detection (LODs) of each substrate were comparable, the coefficients of variation (CVs) were found to be smaller using the TMB-based substrate and thus TMB was selected for continued assay development. As shown in Figure 2, ZIKV is captured by 4G2 antibodies that are coated on the surface of the microtiter plate wells. This antibody has been developed previously and is capable of binding to many flaviviruses promiscuously.[33] Once the ZIKV is captured, one of three ZIKV-specific antibodies that we identified is able to also bind to the ZIKV in a highly selective manner, forming the top half of the ZIKV sandwich. If the virus is present and P1F12, P1H90, or P1B04 bind, an HRP-conjugated anti-rhesus secondary antibody binds to the ZIKV-specific primary antibodies. A signal is then produced as a result of the HRP oxidizing the TMB substrate. This assay for the acute phase detection of ZIKV was performed using spiked samples due to the extreme rarity in obtaining patient samples with active ZIKV. Given that the majority of patients are asymptomatic in the acute phase and, thus, not aware that they are infected, it is very difficult to obtain samples from acutely infected patients. Additionally, these patients who are symptomatic often do not realize that they have been exposed to ZIKV and the short window of time in which the active virus is in circulation is often missed. The urgency of detecting active virus arises when considering the serious negative health consequences generated when pregnant women are infected with ZIKV during their first trimester of pregnancy, with each day playing a critical role in fetal development.[34] Additionally, for future translation of this assay to a point-of-care test, the assay would be invaluable for its ability to screen a large number of individuals in low-resource Zika-epidemic areas around the world. This capability would provide critical data about the ZIKV itself, as well as its detection windows and methods of spread, which are important considerations in the epidemiology of infection with potential epidemic consequences.

A checkerboard assay was employed to optimize the concentrations of the primary and secondary antibodies and thus improve the overall sensitivity. Briefly, on a microtiter plate, the concentrations of primary and secondary antibodies were varied with respect to each other and the assay was run normally. Then, for each antibody pair, the signal-to-noise ratio was calculated by dividing the absorbance value of the signal when the ZIKV is present (+ control) to the absorbance value of the signal when the ZIKV is not present (− control). The results of the checkerboard assay are presented in Figure 3 as a heat map showing the signal-to-noise ratios from low (green) to high (red). The optimal concentrations were chosen by selecting the location on the heat map, where the signal-to-noise ratio was at a maximum. The primary concentrations chosen for P1F12, P1H09, and P1B04 were 0.5, 1.0, and 0.5 μg/mL, respectively, and the secondary concentration chosen was 0.125 μg/mL for all three antibodies.

Next, we optimized the best blocking buffer for our assay. Briefly, our assay was run using different blocking buffers. The results indicated that a protein-free blocking buffer had the highest signal-to-noise ratio. The subsequent optimization steps are summarized in Figure 4: we optimized the incubation times for the antigen, primary and secondary antibodies, as well as the incubation time for the substrate for HRP, TMB. We chose the time points such as to optimize the sensitivity of the assay while minimizing the overall assay time. For this purpose, we arbitrarily set as a condition that at least 80% of the maximum signal was observed. These optimization studies demonstrated that all three primary antibodies reach 80% of the maximum signal within a 120 min period. Likewise, the signal for the secondary antibody was within 80% of its maximum value within 90 min. A 15 min incubation time was sufficient for the substrate TMB. On the other hand, incubation of the antigen never reached a plateau during the optimization time period. Therefore, we selected 120 min as the time for incubation to keep the overall assay time within a relatively short time frame. It should be noted that the assay time can be reduced further with little loss of sensitivity by reducing the incubation times even further. Our total assay time is approximately 6 h. Current gold-standard RT-PCR

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methods take a minimum of 4 h for the PCR portion of the assay. RT-PCR requires sample preparation, including nucleic acid extraction and purification, which adds time to the overall time of the assay. We should note that our assay does not require a sample preparation step and, therefore, is simpler to run than the RT-PCR. In addition, our assay time could be cut down to 4 h or less if desired by reducing the incubation time of the primary antibody at the expense of detection limits, as mentioned.

Our assay was then translated from the Zika incubation buffer to pooled human bodily fluids, including urine, saliva, serum, and whole blood. Figure 5 shows the matrix effect that arose as a result of the incorporation of bodily fluids, outlining the loss in sensitivity due to the antibodies nonspecifically binding to proteins inherently present in human bodily fluids. Neat urine did not demonstrate any significant matrix effect, which increases its appeal as the matrix of choice for the ZIKV immunoassay since it is easily collected from both male and female patients and provides a lengthy 20-day window where ZIKV can be detected. The optimal dilution for the detection of ZIKV in saliva, serum, and whole blood was found to be a 1:10 ratio since it does not sacrifice the sensitivity of the assay while minimizing the matrix effect.

Each step of the assay was optimized to determine the conditions for the assay that yield the lowest LOD of the antigen. Such parameters included the concentrations of the capture antibody and detection antibody, the composition of the blocking solution, as well as the incubation time period for the antigen, capture antibody, detection antibody, secondary antibody, and TMB. The results of these optimization studies resulted in the final version of the immunoassay, as explained in the Materials and Methods part of this manuscript. Using varied concentrations of ZIKV, a calibration curve was generated. The data were fitted to a nonlinear sigmoidal dose–response curve (variable slope) using GraphPad Prism Version 7.02 for Windows (GraphPad Software, La Jolla, CA, USA).

Figure 2. ZIKV assay schematic.

Figure 3. Checkerboard optimization assay for the antibody pairs with the x-axis containing the primary antibody concentrations and the y-axis the secondary antibody concentrations. The results are presented as a heat map plot with reported S/N ratios. Primary antibodies used are (A) P1F12, (B) P1H09, and (C) P1B04. The secondary antibody used in the assay is a preadsorbed, HRP-conjugated goat anti-mouse IgG1 heavy chain.
The LOD and limit of quantitation (LOQ) values were interpolated from the calibration curves using LOD = \( S_B + 3 \times S_{DB} \) and LOQ = \( S_B + 10 \times S_{DB} \), where \( S_B \) and \( S_{DB} \) are the mean and the standard deviation of the blank measurements, respectively. The coefficients of variations for each concentration of antigen were calculated by running the assay for five consecutive days for each of the three primary antibodies. The assay validation data are summarized in Table 2. Our assay sensitivity is similar in each of the bodily fluid tested, which means that the detection of the ZIKV is about an order of magnitude better in urine since we do not have to dilute urine samples. Our detection limits for each antibody in each bodily fluid range from \((3.73 \pm 0.71) \times 10^4\) for buffer using P1B04 antibody to \((2.05 \pm 2.5) \times 10^5\) copies of ZIKV/mL for P1F12 antibody in whole blood. When we compare our limit of detection values with Table 1, we see that our detection limits fall below the reported ZIKV levels in each of the corresponding bodily fluid.

To determine the accuracy of the assay, we calculated the percent recovery of the ZIKV by spiking known concentrations of ZIKV and calculating the interpolated results back to the concentrations. These experiments were performed on three separate days with three separate plates. We have calculated the % recovery for three different concentrations of the ZIKV that fall within the linear range of the assay. The concentrations were selected so that we can calculate the accuracy of the assay when the ZIKV levels are close to the detection limit \((7.5 \times 10^4\) copies ZIKV/mL), fall in the middle of the linear range \((4.0 \times 10^5\) copies ZIKV/mL), and the final concentration was selected so it is close to the high end of the linear portion of the assay \((4.0 \times 10^6\) copies ZIKV/mL). Our worst recovery, obtained using diluted saliva and measured using P1F12, which is the lowest performing antibody of the three, is still approximately 76% of the actual ZIKV concentration, bordering the ±20% clinically accepted values for percent recovery.\(^5\) Out of the 15 different combinations of the 5 human bodily fluids and three different antibodies, 13 fall within the clinically accepted values and the remaining two deviate only 1.3 and 4.1%. At the concentrations where the ZIKV copy numbers are higher, our assay predicted the
Table 2. Assay Parameters for Each ZIKV Selective Antibody in Various Bodily Fluids

| Buffer          | Urine neat | saliva 1:10 | serum 1:10 | whole blood 1:10 |
|-----------------|------------|-------------|------------|------------------|
| P1F12 LOD (copies/mL) | (1.1 ± 1.4) × 10^6 | (8.5 ± 5.6) × 10^6 | (7.4 ± 2.4) × 10^6 | (5.8 ± 0.3) × 10^6 | (2.1 ± 2.5) × 10^6 |
| LOQ (copies/mL)  | (3.0 ± 3.7) × 10^5 | (1.7 ± 1.5) × 10^6 | (1.7 ± 0.98) × 10^6 | (1.1 ± 0.20) × 10^6 | (4.1 ± 5.2) × 10^6 |
| CV Min (%)       | 5.5 ± 3.9 | 3.2 ± 1.5 | 8.7 ± 6.7 | 2.1 ± 2.7 | 3.3 ± 2.1 |
| CV Med (%)       | 6.1 ± 2.4 | 7.4 ± 8.0 | 4.1 ± 9.0 | 4.0 ± 2.9 | 5.8 ± 1.7 |
| CV Max (%)       | 2.1 ± 0.46 | 2.5 ± 1.8 | 1.3 ± 1.2 | 1.8 ± 0.46 | 4.7 ± 3.4 |
| recovery Min (%) | 90.99 ± 14.0 | 94.92 ± 2.15 | 75.89 ± 34.8 | 87.4 ± 6.5 | 92.41 ± 11.1 |
| recovery Med (%) | 86.23 ± 24.9 | 78.70 ± 8.84 | 102.42 ± 24.6 | 105.35 ± 57.8 | 92.20 ± 2.5 |
| recovery Max (%) | 92.20 ± 25.9 | 95.10 ± 20.6 | 112.83 ± 11.2 | 105.83 ± 6.4 | 96.71 ± 3.5 |
| P1H09 LOD (copies/mL) | (3.8 ± 0.72) × 10^8 | (4.4 ± 6.0) × 10^6 | (5.5 ± 6.6) × 10^6 | (5.6 ± 6.6) × 10^6 | (5.5 ± 8.1) × 10^6 |
| LOQ (copies/mL)  | (5.1 ± 1.5) × 10^7 | (4.7 ± 0.6) × 10^6 | (7.1 ± 0.06) × 10^6 | (7.6 ± 1.5) × 10^6 | (7.6 ± 1.0) × 10^6 |
| CV Min (%)       | 5.7 ± 7.6 | 4.2 ± 2.7 | 5.9 ± 7.2 | 3.6 ± 2.3 | 4.7 ± 2.0 |
| CV Med (%)       | 2.5 ± 1.1 | 13.3 ± 9.9 | 4.3 ± 2.0 | 5.1 ± 3.2 | 3.6 ± 1.0 |
| CV Max (%)       | 1.1 ± 0.75 | 1.7 ± 0.56 | 1.0 ± 0.71 | 2.9 ± 3.5 | 0.85 ± 0.44 |
| recovery Min (%) | 95.25 ± 14.2 | 98.90 ± 19.2 | 81.08 ± 32.0 | 75.73 ± 4.6 | 101.71 ± 2.6 |
| recovery Med (%) | 102.53 ± 29.3 | 97.29 ± 29.4 | 98.76 ± 20.2 | 88.57 ± 15.2 | 92.96 ± 4.5 |
| recovery Max (%) | 105.87 ± 12.5 | 109.9 ± 3.72 | 118.45 ± 8.33 | 103.87 ± 11.9 | 94.18 ± 0.6 |
| P1H04 LOD (copies/mL) | (3.7 ± 0.71) × 10^7 | (5.6 ± 0.90) × 10^8 | (4.7 ± 0.64) × 10^4 | (5.9 ± 0.50) × 10^4 | (5.0 ± 0.8) × 10^7 |
| LOQ (copies/mL)  | (4.5 ± 1.8) × 10^6 | (6.2 ± 1.7) × 10^6 | (5.2 ± 0.94) × 10^4 | (7.2 ± 0.91) × 10^4 | (6.5 ± 0.9) × 10^7 |
| CV Min (%)       | 2.6 ± 1.4 | 7.5 ± 2.5 | 7.7 ± 2.5 | 1.6 ± 1.0 | 7.8 ± 2.9 |
| CV Med (%)       | 2.3 ± 2.1 | 4.7 ± 1.5 | 7.0 ± 8.3 | 3.6 ± 2.3 | 4.1 ± 2.8 |
| CV Max (%)       | 1.0 ± 0.24 | 0.49 ± 0.15 | 1.4 ± 0.78 | 0.95 ± 0.35 | 0.97 ± 0.31 |
| recovery Min (%) | 90.65 ± 15.3 | 103.9 ± 15.0 | 90.84 ± 15.8 | 83.93 ± 12.1 | 96.09 ± 5.0 |
| recovery Med (%) | 93.67 ± 15.2 | 103.6 ± 16.9 | 97.74 ± 16.6 | 93.55 ± 5.0 | 90.33 ± 5.6 |
| recovery Max (%) | 102.06 ± 11.1 | 117.0 ± 6.56 | 106.28 ± 5.0 | 95.36 ± 10.9 | 89.89 ± 5.0 |

Concentration very well, around 100%. These results indicate a very high accuracy for our ZIKV assay.

We have also calculated the precision of our assay by measuring the same three different concentrations that were described above in quadruplicates on three separate days and calculating the CV for each antibody, at each concentration for each bodily fluid. CV Min, CV Med, and CV Max correspond to concentrations of 7.5 × 10^5, 4.0 × 10^5, and 7.5 × 10^5 copies ZIKV/mL, respectively. Recovery Min, recovery Med, and recovery Max also correspond to the same concentrations. Correspondingly, a receiver operating characteristic curve could not be generated.

**MATERIALS**

**Reagents.** The following reagents were purchased from Becton Dickinson (Franklin Lakes, NJ): allophycocyanin (APC)—cyanine (Cy7) anti-human CD19 (clone SJ25C1), phycoerythrin (PE)-CF594 anti-human CD3 (clone UCHT1), PE-CF594 anti-human CD14 (clone MAbP9), fluorescein isothiocyanate (FITC) anti-human CD38 (clone HB7), peridinin chlorophyll protein complex (PerCP) anti-human CD20 (clone L27), and PE anti-human CD138 (clone MI15). Ficol–Paque was purchased from GE Lifesciences (Pittsburgh, PA). APC anti-human CD27 antibody (clone O323) was obtained from Biolegend (San Diego, CA). Inactivated Brazilian Fortaleza strain of the Zika virus and the primary anti-ZIKV antibodies were kindly provided by Dr. David Watkins. Magnesium chloride, sodium phosphate dibasic, potassium phosphate monobasic, sodium chloride, potassium chloride, ethylene diamine tetraacetic acid (EDTA), dithiothreitol (DTT), IGEPAL CA-630, Tris HCl, Tween 20, and sodium bicarbonate were purchased from Sigma-Aldrich (St. Louis, MO). LIVE/DEAD Fixable Red Dead Cell Stain Kit, yeast tRNA, IDT, dNTP, SuperScript III Reverse Transcriptase, Molecular Biology Grade water, Expo293F human cell lines, Protein A Plus columns, ExpoPectamine 293 Transfection kit (Cat A14525), SuperSignal ELISA Pico Chemiluminescent Substrate (Cat 37070), Pierce 1-Step Ultra TMB-ELISA.
solution (Cat 34028), Starting Block (PBS) Blocking Buffer (Cat 37539), 3% BSA-Blocker BSA (10%) in PBS (Cat 37525), Blocker BLOTTO in TBS (Cat 37530), and Super Block T20 (PBS) Blocking Buffer (Cat 37516) were purchased from Thermo Fisher Scientific (Waltham, MA). Mouse-anti-flavivirus monoclonal antibody 4G2 (clone D1-4G2-4-15) was purchased from EMD Millipore (Billerica, MA), and a 20 U RNase inhibitor was purchased from New England Biolabs (Ipswich, MA). HotStarTaq Plus DNA polymerase was purchased from Qiagen (Hilden, Germany). Recombinant Zika E protein (MBSS96001) was purchased from MyBioSource (San Diego, CA). Goat anti-human IgG ads-HRP (2045-05) was purchased from SouthernBiotech (Birmingham, AL). The TMB Stop Solution (Cat 50-85-06) was purchased from KPL (Milford, MA). Protein-free blocking buffer (Cat 786-665) was purchased from G Biosciences (St. Louis, MO). Pooled normal human urine (Cat IR100007P), serum (Cat IPLA-SER), whole blood (Cat IPLA-WB1), and saliva (Cat IR100044P) were purchased from Innovative Research Inc. (Novi, MI). Milk was purchased from a local grocery store. 

The Zika incubation buffer was composed of 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.05% Tween 20, 1.0% BSA, and 2% poly(ethylene glycol) pH 7.40. The wash buffer was composed of 100 mM NaHCO₃, pH 9.60. The wash buffer was composed of 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, 137 mM sodium chloride, 2.7 mM potassium chloride, 0.1% Tween 20, pH 7.40.

**Apparatus.** For all experiments, commercial, high-binding polystyrene microtiter plates (Cat 1504), purchased from Thermo Fisher Scientific (Waltham, MA), were used. The washing steps were performed using Molecular Devices (Sunnyvale, CA) MultiWash+ Plate washer using five cycles of 250 μL/well of wash buffer, employing a 10 s shaking step at the end of each cycle. Absorbance measurements were performed on a Clariostar Optima UV/Vis Spectrophotometer (BM Labtech, Ortenberg, Germany). FACS-Aria Ilu flow cytometer was purchased from Becton Dickinson (Franklin Lakes, NJ), and FlowJo was purchased from FlowJo (Ashland, OR). Nanodrop device was purchased from Thermo Fisher Scientific (Waltham, MA).

**METHODS**

**Identification of Zika-Specific Antibodies. Patients.** Blood and urine samples were collected from three volunteers reporting a pruriginous skin rash from São Paulo, Brazil. A real-time reverse transcriptase PCR assay was run on these samples to confirm the presence of ZIKV RNA. Two of the three patients reported a previous history of yellow fever immunization and dengue infection, while the third patient had no history of dengue or yellow fever vaccination. Peripheral blood mononuclear cells (PBMCs) were obtained from blood samples collected between 8 and 13 days post onset of symptoms.

**Flow Cytometry/Plasmablast Sorting.** The frequency of plasmablasts in circulation was determined by flow cytometric analysis of PBMCs obtained from blood collected in acid citrate dextrose using a Ficoll–Paque gradient. Fresh PBMC samples (1 × 10⁶ cells, room temperature, in the dark) were stained with 100 μL of a cocktail containing the following fluorophore–antibody conjugates: phycoerythrin (PE)-CF594 anti-human CD3 (clone UCHT1), PE-CF594 anti-human CD14 (clone MφP9), PE anti-human CD138 (clone M115), allophyocyanin (APC)–cyanine (Cy)7 anti-human CD19 (clone SJ25C1), fluorescein isothiocyanate (FITC) anti-human CD38 (clone HB7), APC anti-human CD27 antibody (clone O323), and peridinin chlorophyll protein complex (PerCP) anti-human CD20 (clone L27). To discriminate between live and dead cells, we included the fixable viability dye LIVE/DEAD Fixable Red Dead Cell Stain Kit in the staining mix. After a 30 min incubation period, the cells were washed twice with FACS buffer (PBS, 0.5% FBS, 2 mM EDTA). Next, the cells were resuspended with a PBS 1× solution and stored at 4 °C until the flow cytometry was performed later the same day using BD FACSaria II flow cytometer and analyzed with FlowJo 9. Flow cytometry identified a population of CD3− CD14− CD19+ CD20− CD38+ CD27+ cells. The same flow cytometry analysis was performed on PBMC samples (5 × 10⁶ cells). Single plasmablast cells were sorted into 96-well plates containing a lysis buffer designed to extract and preserve the RNA (375 mM KCl, 250 mM Tris–HCl pH 8.3, 15 mM MgCl₂, 6.25 mM DTT, 20 U RNase inhibitor, 250 ng/well yeast tRNA, 0.0625 μL/well IGEPAL CA-630). The RNA plates were immediately frozen in dry ice after sorting for subsequent cloning of the Ab chains.

**Ab Repertoire Analysis.** Reverse transcription and a nested PCR were carried out to amplify the variable region of the Ig chains using established protocols. In brief, cDNA was synthesized in a 25 μL reaction using the original sort plates. Each reaction had 2 μL of 10 mM dNTP, 1 μL of 150 ng random hexamer (IDT), 1 μL of SuperScript III Reverse Transcriptase, 20 μL of single-sorted cell sample in lysis buffer, and 1 μL of molecular biology grade water as described above. The reverse transcription reaction was performed at 42 °C for 10 min, 25 °C for 10 min, 50 °C for 60 min, and 94 °C for 10 min and the cDNA was stored at −20 °C after completion. Three different nested PCR reactions, light and heavy chains, were amplified using a HotStarTaq Plus DNA Polymerase and a mix of S′ V-specific primers with matching S′ primers to the constant regions of IgG, immunoglobulin lambda (IgL), and immunoglobulin kappa (IGK). The second PCR reactions were carried out with primers redesigned to incorporate restriction sites compatible with subcloning into rhesus IgG expression vectors instead of the original human vectors. We sequenced the amplified and cloned products using primers complementary to the Ig constant regions, which were subsequently analyzed using IgBLAST to identify SHM levels, as well as V(D)J gene rearrangements.

**Expression and Purification of the Antibodies.** The mAbs were expressed in Expi293F human cell lines. Cotransfection of the plasmids encoding the genes for the antibody’s light and heavy chains was performed using the ExpiFectamine 293 Transfection kit. The secreted mAb was harvested from the supernatant after 5–6 days and the Ig concentration in the supernatant was determined by an anti-rhesus IgG ELISA. A protein A column was employed to purify the antibody and the concentration was determined using the NanoDrop.

**Virus Capture Assay and Recombinant E Protein ELISA.** Side-by-side ZIKV virus capture assay (VCA)/recombinant (r)E ELISA was used to determine antibody binding. Half of the ELISA plate was coated with the mouse-
anti-flavivirus monoclonal antibody 4G2 (clone D1-4G2-4-15) diluted 1:1000 in carbonate binding buffer and half of the plate was coated with recombinant Zika E protein diluted to 5 μg/mL in PBS and incubated overnight at 4 °C. The plate was washed five times with PBS-Tween 20, and the wells were blocked with 5% skim milk in PBS for 1 h at 37 °C. The plate was then washed again and whole virus or E and PBS were added to corresponding VCA and E wells, respectively, and incubated for 1 h at room temperature. The mAb samples diluted to 1 μg/mL were added to the washed plate and incubated for 1 h at 37 °C. Following sample addition, detection was carried out by employing the antibody goat anti-human IgG ads-HRP (1:10 000 dilution). After a wash step, TMB substrate was added and incubated at room temperature for 3–4 min. The reaction was then stopped using TMB stop solution, and the absorbance was read at 450 nm.

**Selectivity.** The selectivity of the assay was performed against different strains of dengue viruses (DENV1, DENV2, DENV3, and DENV4). The wells of a 96-well plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. Next, 100 μL of different strains of DENV were added at varying concentrations from 5.5 × 10^5 to 1.3 × 10^6 copies/mL in Zika incubation buffer and incubated overnight at 4 °C. After a wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 0.5, and 0.125 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 0.5, 0.0625, and 0.125 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 1 h at 37 °C. Next, 100 μL of the secondary Ab was added at varying concentrations from 0.97 ng/mL to 2 μg/mL in Zika incubation buffer, was added across the rows and incubated at room temperature for 2 h. Following another wash step, 100 μL/well of the primary Ab, at varying concentrations from 0.97 ng/mL to 2 μg/mL in Zika incubation buffer, was added across the columns and incubated at room temperature for 1 h. The plate was washed again, and 100 μL/well of TMB was added and incubated for 10 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

**Optimization of HRP Substrate.** The HRP substrate was optimized using a luminol-based chemiluminescent substrate and TMB, which is an absorbance-based substrate. For this purpose, the wells of two 96-well microtiter plates, plate A and plate B, were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plates were washed using wash buffer, and subsequently an aliquot of 100 μL of ZIKV antigen was added at a concentration of 1 × 10^6 copies/mL in triplicates in Zika incubation buffer for 2 h at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer for 2 h at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated for 1 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for 5 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

**Optimization of Antibody Pairs.** Proper primary and secondary antibody concentrations were optimized by titrating each antibody on a microtiter plate (checkerboard assay). For this purpose, the wells of a 96-well plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of Starting Block overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen at a concentration of 1 × 10^6 copies/mL in Zika incubation buffer was added to odd-numbered wells, and a blank solution was added to the even-numbered wells and incubated at room temperature for 2 h. Following another wash step, 100 μL/well of the primary Ab, at varying concentrations from 0.97 ng/mL to 2 μg/mL in Zika incubation buffer, was added across the rows and incubated at room temperature for 2 h. Following another wash step, 100 μL/well of the secondary Ab, at varying concentrations from 78 ng/mL to 10 μg/mL in Zika incubation buffer, was added across the columns and incubated at room temperature for 1 h. The plate was washed again, and 100 μL/well of TMB was added and incubated for 10 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

**Optimization of Blocking Conditions.** To determine which blocking buffer performed best during the blocking step, different commercially available blocking buffers were tested (Starting Block, 3% BSA, protein-free blocking buffer, Blotto Block, Tween 20, 3% milk, Super Block, and PBS as negative control). For this purpose, the wells of a 96-well microtiter plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of one of the seven blocking buffers, or PBS as negative control, overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen was added at a concentration of 1 × 10^6 copies/mL in triplicates in Zika incubation buffer for 2 h at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated for 1 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for 5 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

**Optimization of the Incubation Time for ZIKV Antigen.** The optimum incubation time for the antigen was determined by coating the wells of a 96-well plate with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of protein-free blocking buffer overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen was added into each well in triplicates at a concentration of 1 × 10^6 copies/mL in Zika incubation buffer for varying time intervals between 15 and 180 min at room temperature. Following another wash step, an aliquot of 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer.
and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated for 1 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for 10 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

Optimization of the Incubation Time for the Primary Antibodies. The optimum incubation time for the primary antibody was determined. The wells of a 96-well plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of protein-free blocking buffer overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen at a concentration of 1 × 10^6 copies/mL in Zika incubation buffer was added and incubated for 90 min at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for varying time intervals between 15 and 180 min. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated at room temperature for 1 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for 10 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

Optimization of the Incubation Time for the Secondary Antibody. The secondary antibody incubation time was optimized by coating the wells of a 96-well microtiter plate with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of protein-free blocking buffer overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen was added at a concentration of 1 × 10^6 copies/mL in Zika incubation buffer and incubated for 90 min at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for varying time intervals between 15 and 180 min. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 μg/mL of 4G2 anti-mouse IgG2a antibody in coating buffer overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of 4G2 anti-mouse IgG2a antibody in coating buffer was added and incubated for 90 min at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 1 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for 10 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

Optimization of the Incubation Time for TMB. The final incubation time optimization was performed for TMB incubation step. The wells of a 96-well microtiter plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of protein-free blocking buffer overnight at 4 °C. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen was added at a concentration of 1 × 10^6 copies/mL in Zika incubation buffer for 90 min at room temperature. Following another wash step, 100 μL of the primary Ab was added at concentrations of 0.5, 1.0, and 0.5 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL/well of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated at room temperature for 2 h. The plate was washed again, and 100 μL of TMB solution was added and incubated for varying time intervals between 1 and 60 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

Determination of the Matrix Effect. ZIKV can be found in human bodily fluids including urine, blood, serum, plasma, semen, breast milk, and saliva. To test whether our immunoassay could accurately detect the presence of ZIKV in various human bodily fluids, we have purchased serum, saliva, urine, and whole blood from Innovative Research and spiked with various concentrations of ZIKV. The immunoassay was initially run in urine, saliva, whole blood, and serum without any dilution of the matrix, as these would be the ideal conditions in which to detect ZIKV. However, the results revealed the presence of a matrix effect with the saliva, serum, and whole blood. The assay was repeated using 5- to 100-fold dilutions of the saliva, serum, and whole blood in Zika incubation buffer.

Immunoassay Procedure. The immunoassay procedure was carried out via a sandwich-format colorimetric immunoassay in human bodily fluids, specifically neat human urine, 10-fold dilutions of human saliva, whole blood, and serum in Zika incubation buffer. For this purpose, the wells of a 96-well, clear bottom, high-binding polystyrene microtiter plate was coated with 100 μL of 1.0 μg/mL pan-flaviv 4G2 anti-mouse IgG2a antibody in coating buffer overnight at 4 °C. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL/well of protein-free blocking buffer overnight at 4 °C. The remaining portions of the procedure were carried out at room temperature. The plate was washed using the same washing procedure, and 100 μL of ZIKV spiked in various human bodily fluids at various concentrations was added into the wells and incubated for 2 h. The plate was washed again, and an aliquot of 100 μL of the primary antibody in Zika incubation buffer was added at the following concentrations: 0.5 μg/mL for P1F12, 1.0 μg/mL for P1H09, and 0.125 μg/mL for P1B04 and incubated for 2 h. The plate was washed again, and 100 μL of HRP-labeled preadsorbed secondary Ab was added at a concentration of 6.25 ng/mL for 1.5 h. Following a wash step, 100 μL of TMB solution was added and incubated for 30 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm.

Assay Validation. Precision. Evaluation of the precision of the assay was performed by repeating the experiment every day for 5 days for each of the three antibodies. The data from these experiments were collected and analyzed to obtain the coefficients of variation for each concentration of antigen. Accuracy. The accuracy of the immunoassay was evaluated via a spike recovery test, which was repeated in separate assays spread over 5 days. To perform the spike recovery test, the wells of a 96-well microtiter plate were coated with 100 μL of 1.0 μg/mL of 4G2 anti-mouse IgG2a antibody overnight at 4 °C.
℃. The wells of the plate were washed using wash buffer and subsequently blocked with 200 μL of protein-free blocking buffer overnight at 4 ℃. The washing step was repeated, and an aliquot of 100 μL of ZIKV antigen was added at varying concentrations from 5.0 × 10^3 to 1.0 × 10^6 copies/mL in Zika incubation buffer, neat urine, 10-fold diluted saliva, serum, and whole blood. These concentrations were used to generate the calibration curve for the immunoassay in their corresponding matrix. On the same plates, 100 μL of ZIKV antigen was added at concentrations that correspond to low, medium, and high matrix. On the same plates, 100 μL aliquots of the analyte (7.5 × 10^4, 4.0 × 10^5, and 7.5 × 10^5 copies/mL) in the same matrix as the calibration curve. The plate was then incubated for 2 h. After the incubation was complete, the plate was washed and 100 μL aliquots of the primary Ab were added at concentrations of 0.5, 1.0, and 0.125 μg/mL for P1F12, P1H09, and P1B04, respectively, in Zika incubation buffer and incubated at room temperature for 2 h. Following another wash step, 100 μL of the secondary Ab was added at a concentration of 6.25 ng/mL in Zika incubation buffer and incubated at room temperature for 90 min. The plate was washed again, and 100 μL of TMB solution was added and incubated for 30 min followed by the addition of a 100 μL aliquot of stop solution to stop the color development. The absorbance measurement for each well was performed using a microtiter plate reader at 450 nm. The calibration curve was generated, and the signals corresponding to low, medium, and high concentrations of the ZIKV were interpolated to establish an assay parameter for accuracy.

Safety. As the ZIKV used to carry out the experiments detailed here was inactivated via irradiation, no unforeseen safety hazards were presented. For the DENV selectivity studies, biosafety level II precautions were employed, and no unanticipated safety hazards were encountered. For future testing of patient samples with active ZIKV and/or DENV, if encountered, appropriate safety proceedings for biosafety level II must be followed.

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Notes

The authors declare no competing financial interest.

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