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Rapid Neutralization Testing System for Zika Virus Based on an Enzyme-Linked Immunospot Assay

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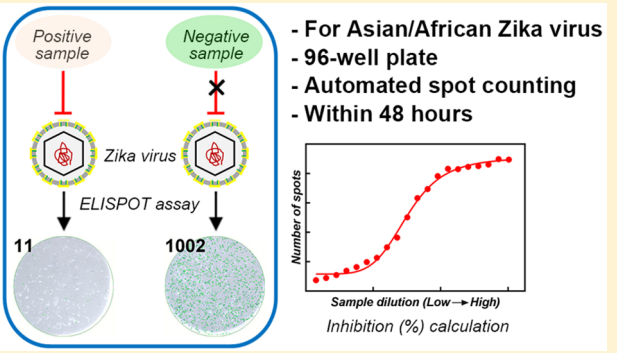
ABSTRACT: Zika virus (ZIKV) is a mosquito-borne flavivirus that has been associated with neuropathology in fetuses and adults, imposing a serious health concern. Therefore, the development of a vaccine is a global health priority. Notably, neutralization tests have a significant value for vaccine development and virus diagnosis. The cytopathic effect (CPE)-based neutralization test (Nt-CPE) is a common neutralization method for ZIKV. However, this method has some drawbacks, such as being time-consuming and labor-intensive and having low-throughput, which precludes its application in the detection of large numbers of specimens. To improve this problem, we developed a neutralization test based on an enzyme-linked immunospot assay (Nt-ELISPOT) for ZIKV and performed the assay in a 96-well format. A monoclonal antibody (mAb), 11C11, with high affinity and reactivity to ZIKV was used to detect ZIKV-infected cells. To optimize this method, the infectious dose of ZIKV was set at a multiplicity of infection (MOI) of 0.0625, and a detection experiment was performed after incubating for 24 h. As a result, under these conditions, the Nt-ELISPOT had good consistency with the traditional Nt-CPE to measure neutralizing titers of sera and neutralizing antibodies. Additionally, three neutralizing antibodies against ZIKV were screened by this method. Overall, we successfully developed an efficient neutralization test for ZIKV that is high-throughput and rapid. This Nt-ELISPOT can potentially be applied to detecting neutralizing titers of large numbers of specimens in vaccine evaluation and neutralizing antibody screening for ZIKV.



KEYWORDS: *Zika virus, ELISPOT, neutralization test, neutralizing antibody*

Zika virus (ZIKV), an arbovirus belonging to the *Flavivirus* genus, is a member of the *Flaviviridae* family. It is closely related to other important arboviruses, such as dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV).[1] ZIKV was first discovered in the serum of a rhesus macaque in Uganda in 1947.[2] Nonetheless, the first ZIKV outbreak occurred on Yap Island in 2007.[3,4] Since then, larger epidemics of ZIKV occurred in French Polynesia in 2013−2014 and in Brazil in 2014−2015.[5] In 2016−2017, ZIKV spread to the Americas where it has become widely disseminated and caused a major epidemic.[6] Increasing evidence indicates the association between ZIKV infection and congenital birth defects and Guillain-Barré syndrome (GBS) in adults.[7−9] In addition, ZIKV could be transmitted through *Aedes* sp. mosquitoes, similar to most other flavivirus members, but also by vertical and sexual routes.[9−11] These unexpected and unique prosperities urgently necessitate a safe and effective vaccine. However, no licensed preventative vaccine or therapeutic drug against ZIKV has been approved.

Humoral immunity response plays a crucial role in the protective mechanism of vaccines. Moreover, the level of neutralizing antibody is considered a significant indicator for epidemiological surveys of ZIKV.[12,13] Therefore, a neutralization assay for ZIKV should be established to promote vaccine development and virus diagnosis. The cytopathic effect (CPE)-based neutralization test (Nt-CPE) has achieved wide use in determining neutralizing titers of serum or antibodies against flaviviruses;[14,15] however, it is not suitable for the detection of large numbers of specimens because of its time-consuming,

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low-throughput, and labor-intensive properties. Therefore, there is an urgent need to develop an efficient neutralization test for ZIKV.

Recently, researchers have tried to develop a high-throughput and rapid neutralization test for ZIKV. In 2017, a rapid ZIKV diagnostic assay (with a turnaround time within 48 h) was reported by Shan et al.[16] However, the luciferase viruses utilized to quantify the neutralizing antibody in their method could not represent the clinical virus strain completely. The enzyme-linked immunospot assay (ELISPOT) has been widely used and demonstrated to be a highly sensitive, objective, and high-throughput immunological method for detecting neutralizing antibodies against many kinds of viruses, including DENV, which is also a flavivirus;[14,17,18] enterovirus 71 (EV71);[19] coxsackievirus A16 (CVA16);[20] coxsackievirus A10 (CVA10);[21] coxsackievirus B3 (CVB3);[22] rotavirus;[23] HSV-1.[24] However, whether this assay can be applied to ZIKV remains unknown. In the present study, a high-throughput and rapid neutralization test based on an ELISPOT assay (Nt-ELISPOT) for ZIKV was established and showed good consistency with the Nt-CPE. Moreover, three neutralizing antibodies against ZIKV were successfully identified by this method.

MATERIALS AND METHODS

Cells and Viruses. African green monkey kidney (Vero) cells (American Type Culture Collection, ATCC) were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37 °C with 5% CO_2.

The three ZIKV strains used in this study were PRVABC59 (GenBank accession No. KU501215, belonging to the Asian lineage ZIKV, isolated from a serum sample of a ZIKV infected patient in 2015, obtained from ATCC),[25] SZ-WIV01 (GenBank accession No. KU963796, belonging to the Asian lineage ZIKV, isolated from a serum sample of a 38-year-old Chinese male patient in 2016, kindly provided by the Academy of Military Medical Sciences of China),[26] and MR766 (GenBank accession No. LC002520, belonging to the African lineage ZIKV, isolated from a sentinel monkey in Uganda in 1947, obtained from ATCC).[27] Viral propagation was performed in Vero cells.[28] For the estimation of viral infectivity titers, virus samples were serially diluted 10-fold (10 dilutions, eight wells for each dilution) and then added to Vero cells (5000 cells/well) preseeded into 96-well plates. After incubating for 5−7 days, cytopathic effects (CPEs) were observed and the 50% tissue culture infectious dose ($TCID_{50}$) of the virus stocks was determined by the Reed-Muench method.[29] For the calculation of multiplicity of infection (MOI), the virus titers were converted from $TCID_{50}$ to plaque forming unit (PFU) by multiplying by 0.69, followed by dividing by the number of cells.

Monoclonal Antibody Production. Monoclonal antibodies (mAbs) against ZIKV were prepared using hybridoma technology.[30] Briefly, six-week-old female BALB/c mice used in this study were purchased from Slac Laboratory Animal Co., Ltd., Shanghai, China, and subcutaneously immunized with the PRVABC59 strain of ZIKV (10^6 $TCID_{50}$/mouse), which was emulsified in an equal volume of Freund's adjuvant (Sigma-Aldrich), three times at 2-week intervals. Then, the virus was infected directly into the spleen of the immunized mice. Three days later, spleen cells were separated and fused with SP2/0 myeloma cells (University of Pavia, Lombardy, Italy) according to hybridoma technology.[30] Afterward, hybridoma supernatants were screened by an enzyme-linked immunosorbent assay (ELISA) against PRVABC59, and the positive clones were further amplified in the abdominal cavity of a mouse. Finally, mAbs were obtained from mouse ascetic fluid and purified by protein A chromatography (GE Healthcare). All the mAbs were conjugated with horseradish peroxidase (HRP) using the $NaIO_4$ oxidation method,[31] mixed with an equal volume of glycerine, and stored at −20 °C.

Western Blotting Assay. Vero cells seeded in 6-well plates were infected with viruses at an MOI of 0.1 or mock infected as a control. Forty-eight hours later, cell lysates were treated with two kinds of loading buffers: one was in a reducing condition with the presence of β-mercaptoethanol (β-ME), and the other was in a nonreducing condition without β-ME. Specifically, the samples with β-ME were heated for 10 min at 100 °C. Then, all the samples were loaded onto a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (Whatman). Subsequently, the proteins were sequentially incubated with an anti-ZIKV mAb (produced in our lab) and an anti-GAPDH mAb (#60004-1-Ig, Proteintech, USA) as the primary antibody, an HRP-conjugated goat-antimouse (GAM-HRP) as the secondary antibody (Sigma-Aldrich, St. Louis, MO, USA), and Pierce ECL Western blotting Substrate (#34095, ThermoFisher Scientific, USA) according to the manufacturer's recommendation.

Immunofluorescence Assay. Vero cells were seeded in 12-well plates containing a 1 cm^2 circular coverslip in each well and then infected with viruses at an MOI of 0.1. At 24 h postinfection, cells on the coverslips were fixed with 4% paraformaldehyde (PFA) in the dark for 30 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min, and blocked with 2% bovine serum albumin (BSA) (in PBS) for 30 min. The cells were then incubated with the anti-ZIKV mAb 11C11 (generated in our laboratory) at 37 °C for 1 h and washed three times with PBS before incubation with Alexa Fluor 488-conjugated secondary antibodies (antimouse IgG, #A21202, Invitrogen) in 2% BSA at 37 °C for 30 min. Finally, after 5 min of DAPI (#D1306, Invitrogen) nuclear staining, cells on the coverslips were imaged with a fluorescence microscope (Zeiss, Germany).

Nt-CPE. Vero cells were seeded with 5×10^3 cells per well into 96-well plates at least 6 h prior to infection. The test samples were serially diluted 2-fold from 1:16 to 1:8192 with DMEM (four wells were used for each dilution). Then, the serial dilutions of test samples were mixed with an equal volume of the PRVABC59 strain of ZIKV (200 PFU) and incubated for 1 h at 35 °C. We chose the low temperature (35 °C) for this experiment to slow the growth of Vero cells so as to have sufficient incubation time to observe the CPE caused by ZIKV infection. The mixtures were then added to the cell plates. The CPE was then observed by microscopy after incubation for 5 days at 35 °C and 5% CO_2. The wells with different dilutions of samples associated with and without CPE were considered as negative and positive wells, respectively. The neutralizing titers were read as the highest dilution that completely inhibited CPE in >50% of the wells.

Positive and Negative Serum/mAb Samples. Positive samples were defined as having an inhibitory effect on the CPE of ZIKV infection. In contrast, negative samples were defined as no inhibition effect on the CPE of ZIKV. Positive and negative serum samples were collected from BALB/c mice that

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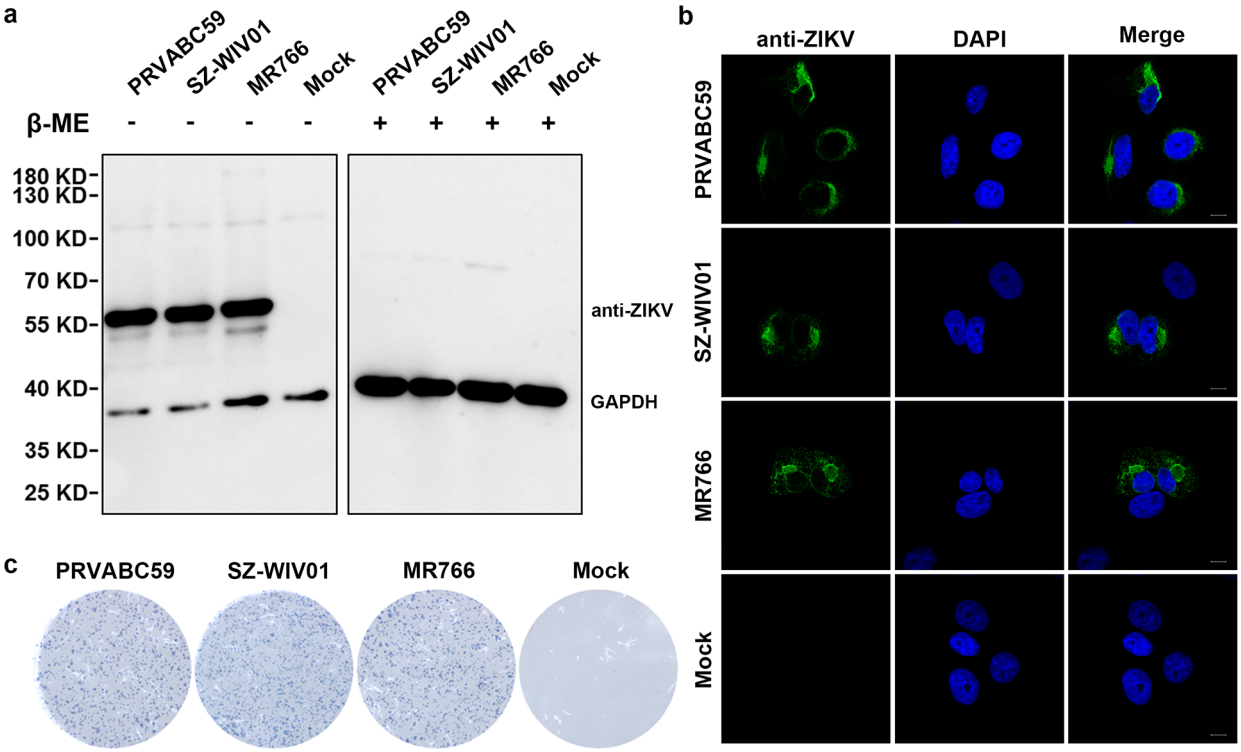

Figure 1. Evaluation of the reactivity of the detection antibody 11C11 against ZIKV. Vero cells infected with three strains of ZIKV (PRVABC59, SZ-WIV01, and MR766) were used in this study. (a) Western blotting analysis. ZIKV-infected cells and mock-infected cells were suspended in lysis buffer with or without the presence of β-mercaptoethanol (β-ME) and subjected to 12% SDS-PAGE. The proteins were probed with the mAb 11C11 and then with a horseradish peroxidase (HRP)-conjugated goat antimouse secondary antibody. (b) Immunofluorescence analysis. ZIKV-infected and mock-infected Vero cells were fixed and incubated with the mAb 11C11 and an Alexa Fluor 488-conjugated antimouse secondary antibody (green). The nuclei were stained with DAPI (blue). Scale bar = 10 μm. (c) ELISPOT assay. PRVABC59-infected (MOI = 0.0625), SZ-WIV01 (MOI = 0.1), MR766-infected (MOI = 0.07), and mock-infected Vero cells were fixed and incubated with the HRP-conjugated mAb 11C11. ZIKV-infected cells were visualized as blue spots after adding TMB substrate.

were immunized and not immunized with the PRVABC59 strain, respectively. The positive mAb sample used in this study was screened by the Nt-CPE test against PRVABC59, and the negative mAb was an unrelated mAb that had no inhibitiory effect on the CPE of ZIKV. All the sera/mAbs were stored at −20 °C, and the serum samples were inactivated at 56 °C for 30 min before testing neutralizing titers.

Nt-ELISPOT. Vero cells were seeded at 2×10^4 cells per well into 96-well plates at least 6 h prior to infection. The test samples were serially diluted 2-fold from 1:16 to 1:8192 with DMEM. Then, the serial dilutions of test samples were mixed with an equal volume of virus in a total volume of 0.1 mL. After incubation for 1 h at 35 °C (to be consistent with the experiments of Nt-CPE, the cells were incubated at 35 °C), the mixtures were added to the cell plates. Specifically, the last two columns of the 96-well plate were set as the virus control column and the cell control column. The virus control wells were infected with the same infectious dose of virus as that for the test wells, and the cell control wells received an equal volume of DMEM. The plates were then incubated at 35 °C for 24 h. After incubation, the cells infected with ZIKV were detected by an ELISPOT assay. Briefly, the cells were fixed with 1% formaldehyde in PBS for 1 h and permeated with 1% Triton X-100 in PBS for 30 min. The detection antibody used for the Nt-ELISPOT, which was conjugated to HRP, was diluted with 2% gelatin and 5% casein (in PBS) and then added into the plates. After incubation for 30 min at 37 °C, the plates were washed five times with PBST (0.05% Tween 20 in PBS). Then, a tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich, St. Louis, MO, USA) was added to the plates and incubated for 10 min. After staining, the plates were patted dry, followed by scanning with an ImmunoSpot 5S UV Analyzer (Cellular Technology Limited, Shaker Heights, OH, USA) using the blue color system, and counted with ImmunoSpot professional analysis software (version 5.0; Cellular Technology Limited). The counting parameters were set as follows: the spot size was set between 0.0001 (Min) and 9.6296 (Max) mm^2, the sensitivity was set to an arbitrary value between 180 and 200, and the background balance and the diffuse spot process was 0. All samples were tested in quadruplicate. The inhibition rate of the test samples was calculated using the following equation: P (%) = $[1 - (N_{\text{test}} - N_{\text{cell control}})/(N_{\text{virus control}} - N_{\text{cell control}})] \times 100\%$. In this equation, P (%) is the inhibition rate of the test sample, and N_{test}, $N_{\text{virus control}}$, and $N_{\text{cell control}}$ are the average number of spots in the test well, in the cell control wells, and in the virus control wells, respectively. The neutralizing titer of the test sample was read as the highest dilution that completely inhibited >50% of the spots.

Statistics. Statistical analyses of experimental data were performed using GraphPad Prism software version 7.04 (GraphPad Prism software Inc., La Jolla, CA). The 50% inhibitory concentration (IC_{50}) of each mAb was defined as the concentration when P (%) equals 50%. The IC_{50} was estimated by nonlinear, dose−response regression analysis. Data are given as the mean ± standard deviation (SD), as

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indicated. Comparisons between two groups were performed using unpaired Student's *t*-tests. A p value of <0.05 was considered statistically significant.

RESULT

Characterization of a Specific Monoclonal Antibody, 11C11, against ZIKV. To select an mAb with high affinity for ZIKV, a group of mAbs against ZIKV were produced in our laboratory from mice that were immunized with the PRVABC59 ZIKV strain. By performing Western blot and immunofluorescence analysis, the mAb 11C11, which exhibited relatively good reactivity with ZIKV-infected (including the Asian lineage ZIKV strains PRVABC59 and SZ-WIV01 and the African lineage ZIKV strain MR766) Vero cells but not with uninfected cells (Figure 1a,b), was selected as the detection antibody for an ELISPOT test. It should be noted that the mAb 11C11 did not react with the samples suspended by reducing loading buffer in the Western blot assay, indicating that 11C11 recognized a conformational region that involves disulfide bonds of a ZIKV protein (Figure 1a). Meanwhile, this recognized region was conserved in both Asian and African lineage ZIKV strains (Figure 1a,b). Then, the mAb 11C11 was further conjugated with HRP (1 mg/mL, 1:1000 dilution rate) to test in the ELISPOT method for recognition of ZIKV-infected Vero cells. As shown in Figure 1c, only the infected cells were visualized as blue spots after adding TMB substrate. The data above indicated that the detection mAb 11C11 could react with both Asian and African lineage ZIKV strains with a low background and showed good affinity and reactivity.

Determination of the Optimal Infectious Dose and Cultivation Periods for the Nt-ELISPOT. The next goal was to establish an effective neutralization test for ZIKV. Since the infectious dose and cultivation periods have a considerable impact on the infection kinetics, we then optimized these factors to develop the Nt-ELISPOT. Because PRVABC59 is a representative virus strain that belongs to the Asian lineage and was isolated from a ZIKV-infected patient in the outbreak in 2015 in Brazil, we selected the PRVABC59 strain of ZIKV for further optimization.

First, an infectious dose between 0.25 and 0.0078125 MOI per well with serial 2-fold dilutions was used to infect Vero cells in this study. Then, during incubation, spot numbers were read and counted every 4 h by the ImmunoSpot analyzer over a period of 40 h. As shown in Figure 2, spots in the plate wells with an MOI = 0.25 or 0.125 could be detected at the incubation time of 16 h and peaked after cultivating for 20 h; however, the spot numbers could not be counted accurately because the spots connected after an incubation time longer than 28 h (so these data were not collected). When the infectious dose was 0.0078125 to 0.0625 MOI, the growth curves of the spot numbers were similar and "S"-shaped; the spot numbers increased with increasing infectious dose and incubation time and then decreased after cultivation for 32 h due to separation of the heavily infected cells from the culture dish. In a previous study, we found that the Nt-ELISPOT for coxsackievirus B3 (CVB3) and coxsackievirus A16 (CVA16) obeys the "percentage law"; that is, when the spots are counted during the log phase or the first half of the stationary phase on the growth curve of the spots, the infectious dose of the Nt-ELISPOT has nearly no influence on the neutralizing titers of the sera.[20,22] Therefore, to shorten the incubation time, we set the infection time to 24 h. At this time point, the higher the infectious dose used, the more spot numbers were counted and

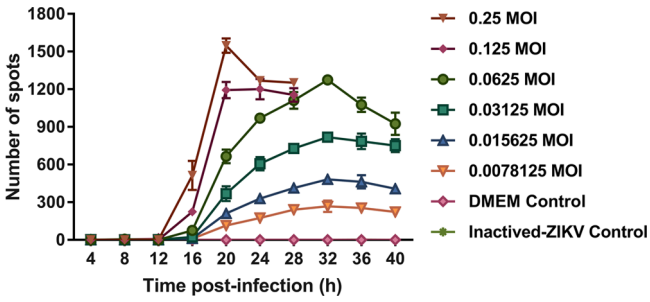

Figure 2. Determination of the optimal infectious dose and cultivation periods for the Nt-ELISPOT. Vero cells (2×10^4 cells/well) preseeded onto 96-well plates were infected with the PRVABC59 strain of ZIKV at an MOI of 0.25 to 0.0078125 per well. An ELISPOT assay was performed to detect the number of spots every 4 h over a period of 40 h. The mock-infected wells and UV-inactivated ZIKV wells were regarded as the negative controls. Each of the dilutions was performed in quadruplicate. The error bars indicate the mean ± SD for the number of spots.

the higher sensitivity was obtained. To balance the relationship between spot numbers and consumption of the virus, an MOI of 0.0625 was selected as the infectious dose for the Nt-ELISPOT. When we chose the infection dose of 0.0625 MOI per well and detection at 24 h postinfection, the number of spots in the infected wells was approximately 1000 but that in the mock-infected group or the UV-inactivated ZIKV group was fewer than 50 (Figure 2), which showed a high reactivity to ZIKV; thus, this infectious dose and infection time were used for the further Nt-ELISPOT. The resulting Nt-ELISPOT protocol for ZIKV is diagrammed in Figure 3. Our approach resulted in a neutralization test that could be completed within 48 h and allowed a 96-well format.

Comparison of the Nt-CPE with the Nt-ELISPOT. To examine the feasibility of the ELISPOT assay in detecting the neutralizing titer of sera and antibody, it was compared with the traditional CPE neutralization test. Four samples (one PRVABC59-immunized mouse serum, one nonimmunized mouse serum, one neutralizing antibody-positive antibody, and one neutralizing antibody-negative antibody) with neutralizing titers of 512, <16, 1024, and <16 for ZIKV, respectively, determined by the Nt-CPE test were used. Furthermore, multiple test samples with decreasing neutralizing capacities were obtained by serially diluting the four samples in 2-fold dilutions and detected by both the Nt-CPE and Nt-ELISPOT. As shown in Figure 4a,c, for the positive samples, the neutralizing titers tested by the Nt-ELISPOT assay decreased 2-fold with the increasing dilution of the samples and had no statistical difference with those determined by the Nt-CPE test. In addition, for the negative samples, the neutralizing titers of the antibody-negative serum or mAb were both <16, as detected by both the Nt-CPE and Nt-ELISPOT (Figure 4b,d). Therefore, these results revealed that the Nt-ELISPOT had good consistency with the Nt-CPE test, which could potentially replace the traditional neutralization test to measure neutralizing titers against ZIKV in both sera and antibodies.

Nt-ELISPOT Could Be Used for the Screening of Neutralizing Antibodies against ZIKV. Neutralizing antibodies are a useful tool for treating disease and studying the pathogenesis of ZIKV.[32,33] Because the Nt-ELISPOT could test the neutralizing titer quickly and with high-throughput, we tried to apply this method to obtain neutralizing antibodies against ZIKV. In detail, the splenocytes from PRVABC59-

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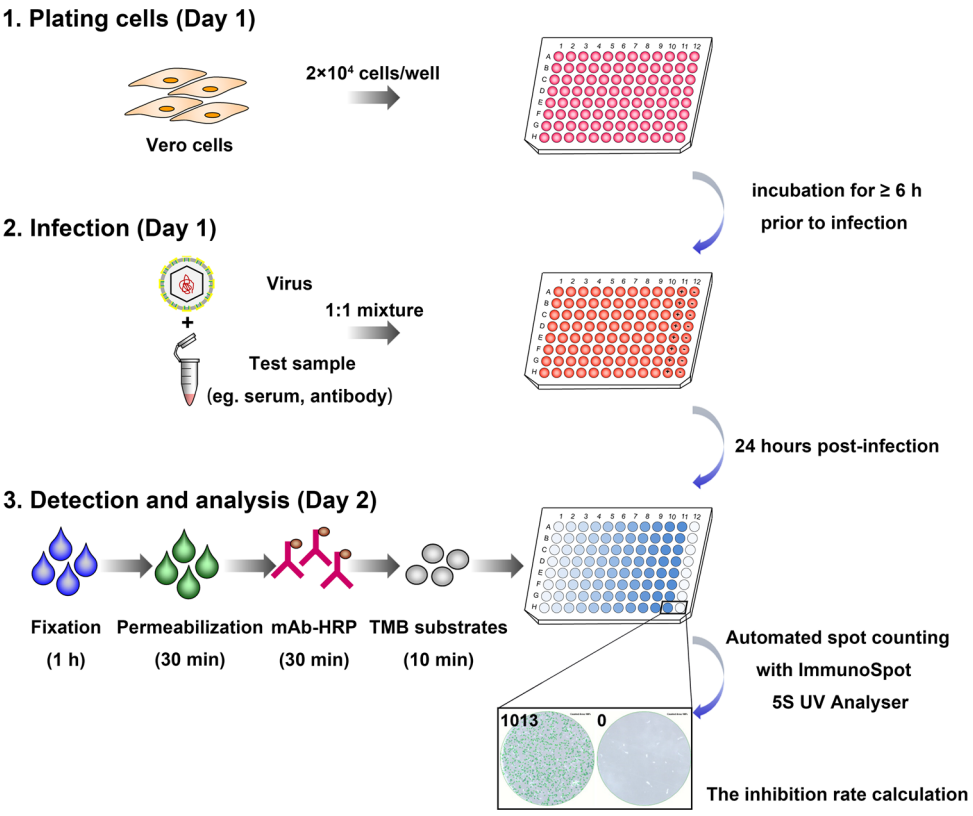

Figure 3. Flow diagram of the ELISPOT assay for the determination of neutralizing titers of the specimens. This assay was performed in a 96-well plate. First, Vero cells (2×10^4 cells/well) were seeded at least 6 h prior to infection. Then, the serial dilutions of the test samples were mixed with an equal volume of virus and incubated for 1 h at 35 °C. The mixtures were added to the cell plates, with the last two columns set as the virus control wells and the cell control wells. After incubation at 35 °C for 24 h, the ELISPOT assay was performed for the detection of neutralizing titers of specimens. The infected wells were visualized as blue spots after adding the TMB substrates. The number of spots was counted by an ImmunoSpot analyzer and recorded in the upper right corner of the image. The neutralizing titer of the test sample was read as the highest dilution that completely inhibited >50% of the spots.

immunized BALB/c mice were fused with sp2/0 cells, and then, the hybridoma supernatant was tested by the Nt-ELISPOT. Eventually, three antibodies were obtained after screening and cloning for five rounds. The results indicated that the mAbs 7F3, 12F12, and 8A6 could effectively inhibit the infection of Vero cells by ZIKV, but the number of spots in the wells of cells treated with an unrelated antibody, which was used in Figure 4d, and PBS-treated cells was >1000 (Figure 5a). To further detect the neutralizing ability of the mAbs, 7F3, 12F12, and 8A6 were serially diluted in a 2-fold ration (the initial concentration was 1 mg/mL) and then tested by the Nt-ELISPOT. As shown in Figure 5b, the IC_{50} values of the mAbs 7F3, 12F12, and 8A6 were 3.55, 1.33, and 0.57 μg/mL, respectively. Therefore, we obtained three neutralizing mAbs against ZIKV as demonstrated by the Nt-ELISPOT.

DISCUSSION

The neutralizing titer of a neutralizing antibody is the key indicator to evaluate the immunogenicity of candidate vaccines.[33] Therefore, a rapid and high-throughput neutralization test for ZIKV is urgently needed, which is significant for promoting the development of vaccines. However, currently, the Nt-CPE assay, the classical neutralization method for flaviviruses,[14,15] is time-consuming (with 5−7 days) and labor-intensive and has low-throughput, so it can hardly meet the demands of the detection of large numbers of specimens in vaccine development or serosurvey. Researchers in this field have tried to overcome this limitation. To our knowledge, two novel neutralization assays for ZIKV have been reported to date. One was based on an ELISA reported in 2016,[34] but the correlation with the traditional neutralization method was unknown, and the whole experimental period needed 4 days, which is nearly as long as the Nt-CPE. The other assay was developed systematically by Shan et al. in 2017,[16] which was rapid and sensitive, but it relied on the virus containing a reporter gene that could not represent the clinical virus strain completely.

In the current study, a neutralization test based on an ELISPOT assay was first developed for ZIKV systematically. ELISPOT technology has been developed for many years. However, it was used initially for analysis of antibody-secreting cells but seldom for humoral immunity assessment.[35] Later, it was applied for detecting cytokines, employing high-affinity capture and detection antibodies.[36] In the past few years, some studies have used it for the detection of neutralizing antibodies against many kinds of viruses (such as EV71, CVA16, CVA10, DENV, HCMV, and so on) due to its highly sensitive, objective, and high-throughput properties.[18−24] Here, to develop the Nt-ELISPOT for ZIKV, several factors were optimized. First, although the Asian lineage of the ZIKV strain was the main lineage in recent outbreaks, the African lineage has also shown some serious clinical signs in animals and human reconstituted tissues.[28,37,38] Therefore, to enhance the applicability of this method, the mAb 11C11, which could

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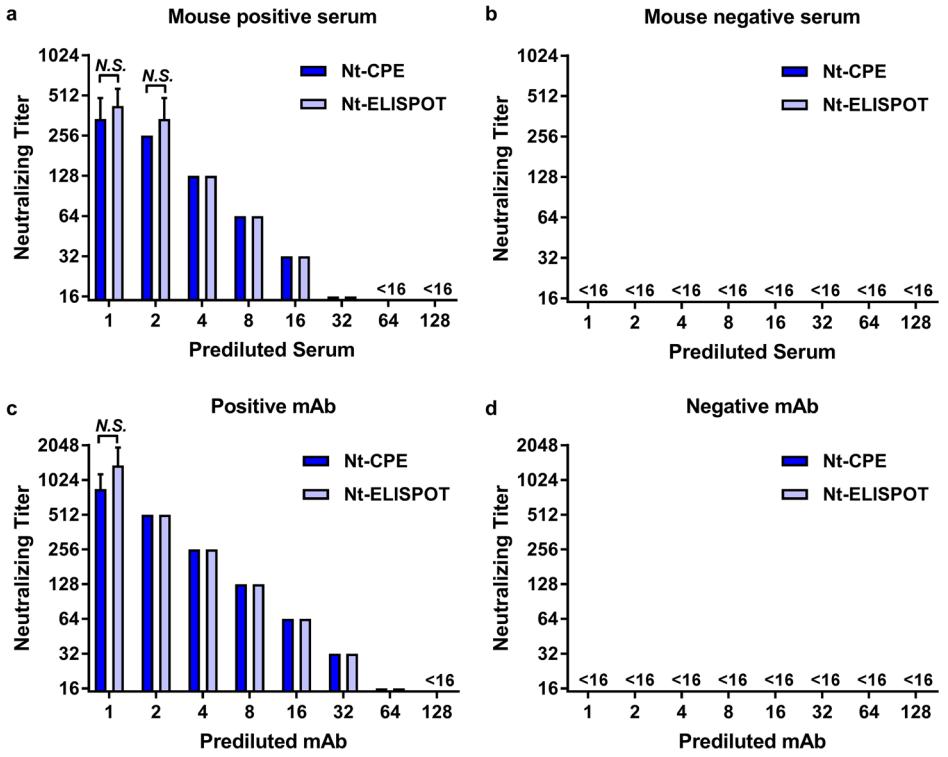

Figure 4. Comparison of the Nt-CPE and the Nt-ELISPOT. Four samples, one ZIKV-immunized mouse serum (a), one nonimmunized mouse serum (b), one neutralizing antibody-positive mAb (c), and one neutralizing antibody-negative mAb (d), selected by the Nt-CPE with the neutralizing titers 512, <16, 1024, and <16, respectively, were serially diluted 2-fold. Each diluted sample was then detected by both the Nt-CPE and Nt-ELISPOT. The neutralizing titer determined by the Nt-CPE and Nt-ELISPOT was read as the highest dilution that completely inhibited CPE in >50% of the wells and completely inhibited >50% of the spots, respectively. All samples were tested in quadruplicate. The error bars indicate the mean ± SD for the neutralizing titer. "N.S." means no significant difference.

react with both Asian and African ZIKV strains with low background and good affinity, was selected as the detection antibody. Then, to shorten the infectious time and obtain clear spots, the optimal infectious dose and incubation time were set as 0.0625 MOI/well and 24 h, respectively. In addition, to verify the feasibility of the ELISPOT assay for the detection of neutralizing titers, we compared the Nt-ELISPOT with Nt-CPE and found that there was a good consistency between them. Overall, the successful construction of the Nt-ELISPOT relied on only a sensitive cell line, a high-affinity antibody, and an automated ELISPOT analyzer. As for the ELISPOT analyzer, it is easy to operate the experiments and capture the ELISPOT images of one 96-well plate. The experiment needs about 5 min/plate. After capturing the images, the number of spots with different sizes can be counted using the ImmunoSpot professional analysis software with the parameter settings (such as sensitivity, background balance, diffuse spot process, and spot separation). Therefore, Nt-ELISPOT could also be further expanded to the application of other viruses.

Indeed, studies from other groups have applied an mAb against ZIKV, such as mAb 4G2 (pan-DENV anti-E protein)[39,40] and a humanized anti-WNV E60,[41] to examine the infected cells in the neutralization tests. First, these antibodies have been used to visualize the ZIKV-infected cell by incubating with HRP-conjugated secondary antibody. In addition, these antibodies were also used for neutralization tests in which a 96-well plate was used for a high-throughput examination. However, the Nt-ELISPOT developed in our study has advantages. First, the antibody used for Nt-ELISPOT had been directly conjugated with HRP, so staining spots need to incubate with only one antibody, which was more convenient and time-saving than the indirect method. Second, the growth curves of the spot numbers with increasing infectious dose and cultivation periods were optimized for the Nt-ELISPOT, so the whole time for detection was less than 48 h. More importantly, the Nt-ELISPOT has good consistency with the traditional Nt-CPE in measuring the neutralizing titers of sera and neutralizing antibodies. Therefore, the neutralization test based on the ELISPOT assay was developed systematically in our study.

The Nt-ELISPOT developed in this study would have a wide application. In addition to evaluating the humoral immune response induced by vaccines, here, we successfully screened three neutralizing antibodies against ZIKV through the use of this method, and the protective efficacy of the mAbs could be further evaluated *in vivo* to promote the study of therapeutic antibodies for ZIKV. Furthermore, this assay could also be used as an assistant tool for seroepidemiological surveys and clinical diagnoses. However, due to serum from humans who may suffer from ZIKV and/or DENV, the background of human serum may be more complex than that of mice serum. Moreover, anti-ZIKV antibodies are highly cross-reactive with other viruses within the same family, so it was challenging to diagnose the cross-reactivity of the serum from ZIKV patients with other flaviviruses, especially DENV.[42] Therefore, in the follow-up study, the human serum samples, especially from ZIKV and DENV infected individuals, needed to be detected by the Nt-ELISPOT to evaluate its clinical relevance. Besides, the reactivity of detection antibody 11C11 with other virus strains also needs to be tested in a further study. If the mAb

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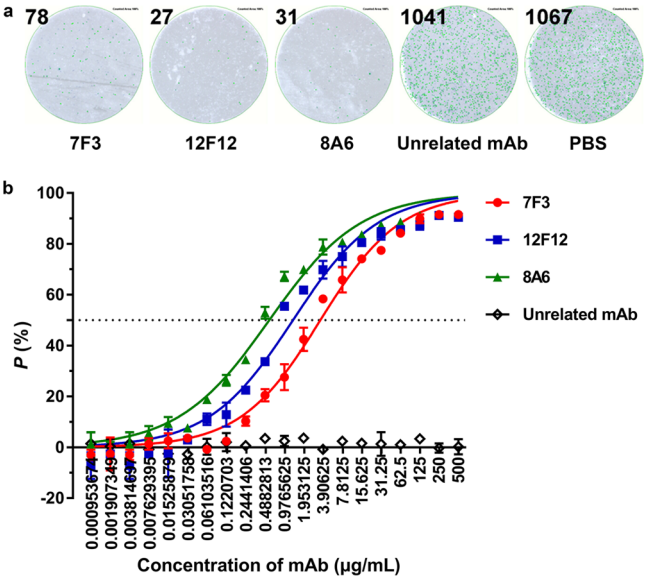

Figure 5. Screening of neutralizing antibodies against ZIKV by the Nt-ELISPOT. (a) Representative well of neutralizing antibodies screened by the Nt-ELISPOT. Three neutralizing antibodies, 7F3, 12F12, and 8A6, were screened by Nt-ELISPOT. Unrelated antibody- and PBS-treated wells were used as controls. The concentration of the mAbs was 125 μg/mL. The ZIKV-infected cells were labeled in blue with HRP-conjugated 11C11. The number of spots was counted by an ImmunoSpot analyzer and recorded in the upper right corner of the image. (b) The IC_{50} of the mAbs 7F3, 12F12, and 8A6 (an initial concentration of 1 mg/mL) detected by the Nt-ELISPOT. Antibodies were diluted serially in 2-fold dilutions. All samples were tested in quadruplicate. Data are given as the mean ± SD as indicated. The IC_{50} was estimated by nonlinear, dose−response regression analysis.

11C11 was specific to ZIKV but not to other common flaviviruses, such as DENV, WNV, and YFV, it might be a useful tool to distinguish ZIKV from other flaviviruses. If it could also react with other flaviviruses, the mAb 11C11 could also be applied as the detection antibody to detect the neutralizing titers against them by Nt-ELISPOT; then, the flavivirus prevalence could be preliminarily understood by the comprehensive analysis of the neutralizing titers for both ZIKV and DENV or other flaviviruses.

In summary, we have successfully developed a neutralization test against ZIKV based on an ELISPOT assay. The Nt-ELISPOT method is high-throughput, objective, and low cost, and the whole detection time was <48 h. Moreover, the technique has a good relationship with the traditional Nt-CPE to measure neutralizing titers of sera or antibodies. Therefore, the Nt-ELISPOT for ZIKV can potentially be applied to detecting neutralizing titers of large numbers of specimens in vaccine evaluation and neutralizing antibody screening of ZIKV.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Lazear, H. M., and Diamond, M. S. (2016) Zika Virus: New Clinical Syndromes and Its Emergence in the Western Hemisphere. *J. Virol. 90* (10), 4864−4875.
(2) Dick, G. W., Kitchen, S. F., and Haddow, A. J. (1952) Zika virus. I. Isolations and serological specificity. *Trans. R. Soc. Trop. Med. Hyg. 46* (5), 509−20.
(3) Duffy, M. R., Chen, T. H., Hancock, W. T., Powers, A. M., Kool, J. L., Lanciotti, R. S., Pretrick, M., Marfel, M., Holzbauer, S., Dubray, C., Guillaumot, L., Griggs, A., Bel, M., Lambert, A. J., Laven, J., Kosoy, O., Panella, A., Biggerstaff, B. J., Fischer, M., and Hayes, E. B. (2009) Zika virus outbreak on Yap Island, Federated States of Micronesia. *N. Engl. J. Med. 360* (24), 2536−43.
(4) Lanciotti, R. S., Kosoy, O. L., Laven, J. J., Velez, J. O., Lambert, A. J., Johnson, A. J., Stanfield, S. M., and Duffy, M. R. (2008) Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerging Infect. Dis. 14* (8), 1232−9.
(5) Musso, D. (2015) Zika Virus Transmission from French Polynesia to Brazil. *Emerging Infect. Dis. 21* (10), 1887.
(6) Faria, N. R., Azevedo, R., Kraemer, M. U. G., Souza, R., Cunha, M. S., Hill, S. C., Theze, J., Bonsall, M. B., Bowden, T. A., Rissanen, I., Rocco, I. M., Nogueira, J. S., Maeda, A. Y., Vasami, F., Macedo, F. L. L., Suzuki, A., Rodrigues, S. G., Cruz, A. C. R., Nunes, B. T., Medeiros, D. B. A., Rodrigues, D. S. G., Queiroz, A. L. N., da Silva, E. V. P., Henriques, D. F., da Rosa, E. S. T., de Oliveira, C. S., Martins, L. C., Vasconcelos, H. B., Casseb, L. M. N., Simith, D. B., Messina, J. P., Abade, L., Lourenco, J., Alcantara, L. C. J., de Lima, M. M., Giovanetti, M., Hay, S. I., de Oliveira, R. S., Lemos, P. D. S., de Oliveira, L. F., de Lima, C. P. S., da Silva, S. P., de Vasconcelos, J. M., Franco, L., Cardoso, J. F., Vianez-Junior, J., Mir, D., Bello, G., Delatorre, E., Khan, K., Creatore, M., Coelho, G. E., de Oliveira, W. K., Tesh, R., Pybus, O. G., Nunes, M. R. T., and Vasconcelos, P. F. C. (2016) Zika virus in the Americas: Early epidemiological and genetic findings. *Science 352* (6283), 345−349.
(7) Zambrano, L. I., Fuentes-Barahona, I. C., Soto-Fernandez, R. J., Zuniga, C., da Silva, J. C., and Rodriguez-Morales, A. J. (2019) Guillain-Barre syndrome associated with Zika virus infection in Honduras, 2016−2017. *Int. J. Infect. Dis. 84*, 136−137.
(8) Brasil, P., Sequeira, P. C., Freitas, A. D., Zogbi, H. E., Calvet, G. A., de Souza, R. V., Siqueira, A. M., de Mendonca, M. C., Nogueira, R. M., de Filippis, A. M., and Solomon, T. (2016) Guillain-Barre syndrome associated with Zika virus infection. *Lancet 387* (10026), 1482.
(9) Mlakar, J., Korva, M., Tul, N., Popovic, M., Poljsak-Prijatelj, M., Mraz, J., Kolenc, M., Resman Rus, K., Vesnaver Vipotnik, T., Fabjan Vodusek, V., Vizjak, A., Pizem, J., Petrovec, M., and Avsic Zupanc, T. (2016) Zika Virus Associated with Microcephaly. *N. Engl. J. Med. 374* (10), 951−8.
(10) Musso, D., and Gubler, D. J. (2016) Zika Virus. *Clin. Microbiol. Rev. 29* (3), 487−524.

DOI: 10.1021/acsinfecdis.9b00333
ACS Infect. Dis. XXXX, XXX, XXX−XXX

(11) Counotte, M. J., Kim, C. R., Wang, J., Bernstein, K., Deal, C. D., Broutet, N. J. N., and Low, N. (2018) Sexual transmission of Zika virus and other flaviviruses: A living systematic review. *PLoS Med. 15* (7), No. e1002611.
(12) Langerak, T., Brinkman, T., Mumtaz, N., Arron, G., Hermelijn, S., Baldewsingh, G., Wongsokarijo, M., Resida, L., Rockx, B., Koopmans, M. P. G., Van Gorp, E. C. M., and Vreden, S. (2019) Zika Virus Seroprevalence in Urban and Rural Areas of Suriname, 2017. *J. Infect. Dis. 220* (1), 28−31.
(13) Netto, E. M., Moreira-Soto, A., Pedroso, C., Hoser, C., Funk, S., Kucharski, A. J., Rockstroh, A., Kummerer, B. M., Sampaio, G. S., Luz, E., Vaz, S. N., Dias, J. P., Bastos, F. A., Cabral, R., Kistemann, T., Ulbert, S., de Lamballerie, X., Jaenisch, T., Brady, O. J., Drosten, C., Sarno, M., Brites, C., and Drexler, J. F. (2017) High Zika Virus Seroprevalence in Salvador, Northeastern Brazil Limits the Potential for Further Outbreaks. *mBio 8* (6), e01390-17.
(14) Li, X. Q., Chen, J., Huang, Y. F., Ding, X. X., Liu, L. D., Qiu, L. W., Pan, Y. X., Deng, Y. Q., Hu, D. M., Di, B., Qin, C. F., and Che, X. Y. (2013) Evaluation and analysis of dengue virus enhancing and neutralizing activities using simple high-throughput assays. *Appl. Microbiol. Biotechnol. 97* (14), 6503−11.
(15) Nurtop, E., Villarroel, P. M. S., Pastorino, B., Ninove, L., Drexler, J. F., Roca, Y., Gake, B., Dubot-Peres, A., Grard, G., Peyrefitte, C., Priet, S., de Lamballerie, X., and Gallian, P. (2018) Combination of ELISA screening and seroneutralisation tests to expedite Zika virus seroprevalence studies. *Virol. J. 15* (1), 192.
(16) Shan, C., Xie, X., Ren, P., Loeffelholz, M. J., Yang, Y., Furuya, A., Dupuis, A. P., 2nd, Kramer, L. D., Wong, S. J., and Shi, P. Y. (2017) A Rapid Zika Diagnostic Assay to Measure Neutralizing Antibodies in Patients. *EBioMedicine 17*, 157−162.
(17) Rodrigo, W. W., Alcena, D. C., Rose, R. C., Jin, X., and Schlesinger, J. J. (2009) An automated Dengue virus microneutralization plaque assay performed in human Fc{gamma} receptor-expressing CV-1 cells. *Am. J. Trop. Med. Hyg. 80* (1), 61−5.
(18) Liu, L., Wen, K., Li, J., Hu, D., Huang, Y., Qiu, L., Cai, J., and Che, X. (2012) Comparison of plaque- and enzyme-linked immunospot-based assays to measure the neutralizing activities of monoclonal antibodies specific to domain III of dengue virus envelope protein. *Clin. Vaccine Immunol. 19* (1), 73−8.
(19) Mao, Q., Cheng, T., Zhu, F., Li, J., Wang, Y., Li, Y., Gao, F., Yang, L., Yao, X., Shao, J., Xia, N., Liang, Z., and Wang, J. (2013) The cross-neutralizing activity of enterovirus 71 subgenotype c4 vaccines in healthy chinese infants and children. *PLoS One 8* (11), No. e79599.
(20) Hou, W., Yang, L., He, D., Zheng, J., Xu, L., Liu, J., Liu, Y., Zhao, H., Ye, X., Cheng, T., and Xia, N. (2015) Development of a coxsackievirus A16 neutralization test based on the enzyme-linked immunospot assay. *J. Virol. Methods 215−216*, 56−60.
(21) Liu, D., Xu, L., Zhu, R., Yin, Z., Lin, Y., Hou, W., Li, S., He, S., Cheng, T., and Xia, N. (2019) Development of an efficient neutralization assay for Coxsackievirus A10. *Appl. Microbiol. Biotechnol. 103* (4), 1931−1938.
(22) Yang, L., He, D., Tang, M., Li, Z., Liu, C., Xu, L., Chen, Y., Du, H., Zhao, Q., Zhang, J., Cheng, T., and Xia, N. (2014) Development of an enzyme-linked immunosorbent spot assay to measure serum-neutralizing antibodies against coxsackievirus B3. *Clin. Vaccine Immunol. 21* (3), 312−20.
(23) Li, T., Lin, H., Yu, L., Xue, M., Ge, S., Zhao, Q., Zhang, J., and Xia, N. (2014) Development of an enzyme-linked immunospot assay for determination of rotavirus infectivity. *J. Virol. Methods 209*, 7−14.
(24) Luo, Y., Xiong, D., Li, H. H., Qiu, S. P., Lin, C. L., Chen, Q., Huang, C. H., Yuan, Q., Zhang, J., and Xia, N. S. (2016) Development of an HSV-1 neutralization test with a glycoprotein D specific antibody for measurement of neutralizing antibody titer in human sera. *Virol. J. 13*, 44.
(25) Lanciotti, R. S., Lambert, A. J., Holodniy, M., Saavedra, S., and Signor, C. (2016) Phylogeny of Zika Virus in Western Hemisphere. *Emerging Infect. Dis. 22* (5), 933−5.
(26) Deng, C., Liu, S., Zhang, Q., Xu, M., Zhang, H., Gu, D., Shi, L., He, J., Xiao, G., and Zhang, B. (2016) Isolation and characterization of Zika virus imported to China using C6/36 mosquito cells. *Virol. Sin. 31* (2), 176−9.
(27) Sabin, A. B. (1952) Research on dengue during World War II. *Am. J. Trop. Med. Hyg. 1* (1), 30−50.
(28) Li, S., Armstrong, N., Zhao, H., Hou, W., Liu, J., Chen, C., Wan, J., Wang, W., Zhong, C., Liu, C., Zhu, H., Xia, N., Cheng, T., and Tang, Q. (2018) Zika Virus Fatally Infects Wild Type Neonatal Mice and Replicates in Central Nervous System. *Viruses 10* (1), 49.
(29) Reed, L. J., and Muench, H. (1938) A simple method of estimating fifty percent endpoints. *Am. J. Epidemiol. 27* (3), 493−497.
(30) Harlow, E., and Lane, D. (1999) *Using antibodies: a laboratory manual*, p xiv, 495 p, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
(31) Nakane, P. K., and Kawaoi, A. (1974) Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem. 22* (12), 1084−91.
(32) Dowd, K. A., DeMaso, C. R., Pelc, R. S., Speer, S. D., Smith, A. R. Y., Goo, L., Platt, D. J., Mascola, J. R., Graham, B. S., Mulligan, M. J., Diamond, M. S., Ledgerwood, J. E., and Pierson, T. C. (2016) Broadly Neutralizing Activity of Zika Virus-Immune Sera Identifies a Single Viral Serotype. *Cell Rep. 16* (6), 1485−1491.
(33) Shan, C., Xie, X., and Shi, P. Y. (2018) Zika Virus Vaccine: Progress and Challenges. *Cell Host Microbe 24* (1), 12−17.
(34) Larocca, R. A., Abbink, P., Peron, J. P., Zanotto, P. M., Iampietro, M. J., Badamchi-Zadeh, A., Boyd, M., Ng'ang'a, D., Kirilova, M., Nityanandam, R., Mercado, N. B., Li, Z., Moseley, E. T., Bricault, C. A., Borducchi, E. N., Giglio, P. B., Jetton, D., Neubauer, G., Nkolola, J. P., Maxfield, L. F., De La Barrera, R. A., Jarman, R. G., Eckels, K. H., Michael, N. L., Thomas, S. J., and Barouch, D. H. (2016) Vaccine protection against Zika virus from Brazil. *Nature 536* (7617), 474−8.
(35) Czerkinsky, C. C., Nilsson, L. A., Nygren, H., Ouchterlony, O., and Tarkowski, A. (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J. Immunol. Methods 65* (1−2), 109−21.
(36) Taguchi, T., McGhee, J. R., Coffman, R. L., Beagley, K. W., Eldridge, J. H., Takatsu, K., and Kiyono, H. (1990) Detection of individual mouse splenic T cells producing IFN-gamma and IL-5 using the enzyme-linked immunospot (ELISPOT) assay. *J. Immunol. Methods 128* (1), 65−73.
(37) Jaeger, A. S., Murrieta, R. A., Goren, L. R., Crooks, C. M., Moriarty, R. V., Weiler, A. M., Rybarczyk, S., Semler, M. R., Huffman, C., Mejia, A., Simmons, H. A., Fritsch, M., Osorio, J. E., Eickhoff, J. C., O'Connor, S. L., Ebel, G. D., Friedrich, T. C., and Aliota, M. T. (2019) Zika viruses of African and Asian lineages cause fetal harm in a mouse model of vertical transmission. *PLoS Neglected Trop. Dis. 13* (4), No. e0007343.
(38) Cagno, V., Tseligka, E. D., Bettex, Q., Huang, S., Constant, S., and Tapparel, C. (2019) Growth of Zika virus in human reconstituted respiratory, intestinal, vaginal and neural tissues. *Clin. Microbiol. Infect. 25*, 1042.e1.
(39) Collins, M. H., McGowan, E., Jadi, R., Young, E., Lopez, C. A., Baric, R. S., Lazear, H. M., and de Silva, A. M. (2017) Lack of Durable Cross-Neutralizing Antibodies Against Zika Virus from Dengue Virus Infection. *Emerging Infect. Dis. 23* (5), 773−781.
(40) Montoya, M., Collins, M., Dejnirattisai, W., Katzelnick, L. C., Puerta-Guardo, H., Jadi, R., Schildhauer, S., Supasa, P., Vasanawathana, S., Malasit, P., Mongkolsapaya, J., de Silva, A. D., Tissera, H., Balmaseda, A., Screaton, G., de Silva, A. M., and Harris, E. (2018) Longitudinal Analysis of Antibody Cross-neutralization Following Zika Virus and Dengue Virus Infection in Asia and the Americas. *J. Infect. Dis. 218* (4), 536−545.
(41) Richner, J. M., Himansu, S., Dowd, K. A., Butler, S. L., Salazar, V., Fox, J. M., Julander, J. G., Tang, W. W., Shresta, S., Pierson, T. C., Ciaramella, G., and Diamond, M. S. (2017) Modified mRNA Vaccines Protect against Zika Virus Infection. *Cell 168* (6), 1114−1125.E10.
(42) Priyamvada, L., Hudson, W., Ahmed, R., and Wrammert, J. (2017) Humoral cross-reactivity between Zika and dengue viruses:

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ACS Infect. Dis. XXXX, XXX, XXX−XXX

implications for protection and pathology. *Emerging Microbes Infect. 6*, 1.

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