Antibody-based assay discriminates Zika virus infection from other flaviviruses

Angel Balmaseda[a,b,1], Karin Stettler[c,1], Raquel Medialdea-Carrera[d], Damaris Collado[b], Xia Jin[c], José Victor Zambrana[b], Stefano Jaconi[c], Elisabetta Cameroni[c], Saira Saborio[a,b], Francesca Rovida[e], Elena Percivalle[e], Samreen Ijaz[f], Steve Dicks[f], Ines Ushiro-Lumb[g], Luisa Barzon[h], Patricia Siqueira[i], David W. G. Brown[i], Fausto Baldanti[e,j], Richard Tedder[f], Maria Zambon[f], A. M. Bispo de Filippis[i], Eva Harris[k,2], and Davide Corti[c,2]

[a]Laboratorio Nacional de Virología, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua 16064, Nicaragua; [b]Sustainable Sciences Institute, Managua 14007, Nicaragua; [c]Humabs BioMed SA, 6500 Bellinzona, Switzerland; [d]National Institute for Health Research Health Protection Research Unit in Emerging and Zoonotic Infections, University of Liverpool, Liverpool L69 3BX, United Kingdom; [e]Molecular Virology Unit, Microbiology and Virology Department, Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo, 27100 Pavia, Italy; [f]Microbiology Services Colindale, Public Health England (PHE), London NW9 5EQ, United Kingdom; [g]Microbiology Services, National Health Service Blood and Transplant, London NW9 5BG, United Kingdom; [h]Department of Molecular Medicine, University of Padova, 35121 Padova, Italy; [i]Fundacão Oswaldo Cruz, Rio de Janeiro CEP 22061-040, Brazil; [j]Department of Clinical, Surgical, Diagnostic, and Pediatric Sciences, University of Pavia, 27100 Pavia, Italy; and [k]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, CA 94720-3370

Edited by Rafi Ahmed, Emory University, Atlanta, GA, and approved June 13, 2017 (received for review March 27, 2017)

Zika virus (ZIKV) is a mosquito-borne flavivirus that emerged recently as a global health threat, causing a pandemic in the Americas. ZIKV infection mostly causes mild disease, but is linked to devastating congenital birth defects and Guillain-Barré syndrome in adults. The high level of cross-reactivity among flaviviruses and their cocirculation has complicated serological approaches to differentially detect ZIKV and dengue virus (DENV) infections, accentuating the urgent need for a specific and sensitive serological test. We previously generated a ZIKV nonstructural protein 1 (NS1)-specific human monoclonal antibody, which we used to develop an NS1-based competition ELISA. Well-characterized samples from RT-PCR-confirmed patients with Zika and individuals exposed to other flavivirus infections or vaccination were used in a comprehensive analysis to determine the sensitivity and specificity of the NS1 blockade-of-binding (BOB) assay, which was established in laboratories in five countries (Nicaragua, Brazil, Italy, United Kingdom, and Switzerland). Of 158 sera/plasma from RT-PCR-confirmed ZIKV infections, 145 (91.8%) yielded greater than 50% inhibition. Of 171 patients with primary or secondary DENV infections, 152 (88.9%) scored negative. When the control group was extended to patients infected by other flaviviruses, other viruses, or healthy donors (n = 540), the specificity was 95.9%. We also analyzed longitudinal samples from DENV-immune and DENV-naive ZIKV infections and found inhibition was achieved within 10 d postonset of illness and maintained over time. Thus, the Zika NS1 BOB assay is sensitive, specific, robust, simple, low-cost, and accessible, and can detect recent and past ZIKV infections for surveillance, seroprevalence studies, and intervention trials.

Zika | serology | flaviviruses | dengue | ELISA

Zika virus (ZIKV) is a mosquito-borne flavivirus that is spread via the bite of infected *Aedes* mosquitoes or by sexual transmission and is responsible for the explosive 2015–2017 epidemic in the Americas. ZIKV infection during pregnancy is linked to devastating birth defects and associated anomalies, designated congenital Zika syndrome (1, 2), whereas in adults, ZIKV infection has been associated with Guillain Barré syndrome (3). Flaviviruses are enveloped RNA viruses containing an ∼11-kb positive-stranded RNA genome that encodes three structural and seven nonstructural proteins. Cells infected by flaviviruses secrete nonstructural protein 1 (NS1), which has multiple roles in immune evasion and pathogenesis (4, 5).

Antibody responses generated in response to flavivirus infections are notoriously cross-reactive, representing a significant obstacle for the specific diagnosis of infection using serological assays. Multiple RT-PCR-based assays for the detection of ZIKV RNA are available, but their use is limited to the narrow window when viral RNA is detectable in body fluids. This is highly variable among patients and subject to reporting error, as symptoms are mild, and thus patients may take less notice of the day of onset, but in most cases, it is up to 7 d in serum, up to 14 d in urine, and more than 20 d in semen (6, 7). Conventional ELISAs using traditional viral antigen have been found to poorly differentiate among flavivirus infections (8, 9). This is especially problematic with antibodies to ZIKV and dengue virus (DENV), which cocirculate in the Americas. Neutralization assays can measure virus-specific neutralizing antibodies; however, specificity is affected by the production of cross-reactive neutralizing antibodies, especially after multiple DENV infections and at early times postinfection (9–11). The lack of accurate serologic methods for identification of ZIKV infection has made it very challenging to determine the burden and rate of asymptomatic infections, define the incidence of congenital Zika syndrome among infected women, and identify neurologic complications associated with ZIKV infection.

In previous studies (10, 12), we identified NS1-reactive monoclonal antibodies (mAbs) derived from ZIKV- and DENV-infected

Significance

Zika virus (ZIKV), a mosquito-borne flavivirus with homology to dengue virus (DENV), has become a public health threat, particularly because of its association with severe congenital birth defects. The high level of cross-reactivity among flaviviruses and their cocirculation has complicated serological approaches to detect ZIKV infections. Thus, there is an urgent need for a specific serological assay to discriminate ZIKV infection from other flaviviruses. This study demonstrates that the antibody-based assay we developed and implemented in five countries has high specificity and sensitivity in the detection of recent and past ZIKV infections. The ZIKV nonstructural protein 1 (NS1) blockade-of-binding ELISA assay is a simple, robust, and low-cost solution for Zika surveillance programs, seroprevalence studies, and intervention trials in flavivirus-endemic areas.

Author contributions: A.B., K.S., E.C., R.T., M.Z., A.M.B.d.F., E.H., and D. Corti designed research; A.B., K.S., R.M.-C., D. Collado, X.J., J.V.Z., S.J., S.S., E.P., S.I., S.D., and P.S. performed research; S.J., F.R., I.U.-L., L.B., and F.B. contributed new reagents/analytic tools; A.B., L.B., F.B., A.M.B.d.F., and E.H. provided samples; A.B., K.S., R.M.-C., D. Collado, X.J., J.V.Z., S.J., E.C., S.S., F.R., E.P., S.I., S.D., I.U.-L., D.W.G.B., R.T., A.M.B.d.F., E.H., and D. Corti analyzed data; and A.B., K.S., D.W.G.B., F.B., M.Z., E.H., and D. Corti wrote the paper.

Conflict of interest statement: K.S, S.J., E.C., X.J., and D. Corti are employees of Humabs Biomed, a company focused on the development of anti-infectives human monoclonal antibodies.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

[1]A.B. and K.S. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: davide.corti@humabs.ch or eharris@berkeley.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1704984114/-/DCSupplemental.

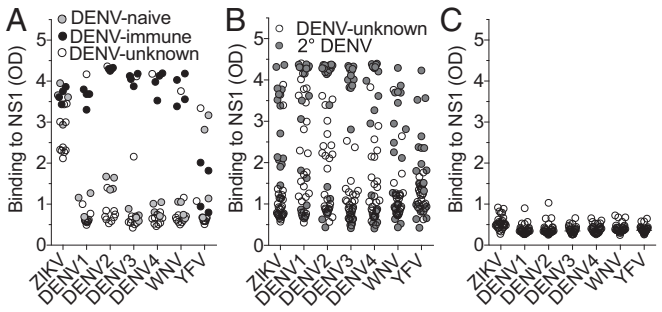

Fig. 1. Binding of plasma IgG derived from ZIKV- and DENV-immune donors to NS1 antigens from different flaviviruses. (*A–C*) Plasma from ZIKV-immune (*A*), DENV-immune (*B*), or heathy (*C*) donors were tested at 1:100 dilution for binding of total IgG to wells coated with ZIKV, DENV1–DENV4, WNV, or YFV NS1. Samples from ZIKV-immune donors known to be DENV-naive, DENV-immune, or unknown are shown in gray, black, and white symbols, respectively. Samples from DENV-immune donors with secondary DENV infections are shown in dark gray.

individuals. The analysis of their cross-reactivity revealed that most of the mAbs induced in subjects exposed to DENV or ZIKV only were virus-specific. Conversely, numerous mAbs isolated from DENV-immune ZIKV-infected donors cross-reacted with both ZIKV and DENV NS1 antigens. Antibody competition studies identified two sites on NS1 that were recognized by ZIKV-specific mAbs and not competed by cross-reactive antibodies (10). One of these mAbs, designated ZKA35 and directed to site S2 on NS1, was used as a probe to develop the serological NS1 blockade-of-binding (BOB) assay described in this study. Here, we established the NS1 BOB ELISA in laboratories in five countries, including Nicaragua and Brazil during the Zika epidemic, and tested a large number of well-characterized samples from RT-PCR-confirmed ZIKV infections, primary and secondary DENV infections, individuals with other flavivirus and other virus infections or vaccinations, and healthy control patients in a comprehensive study to demonstrate the high sensitivity and specificity of the assay and its robust implementation in multiple countries.

Results

Anti-NS1 Antibody Cross-Reactivity Among Different Flaviviruses. Initially, to assess serum reactivity to NS1, plasma from ZIKV-immune ($n = 18$) and DENV-immune ($n = 44$) returned travelers, as well as healthy Swiss blood donors ($n = 30$), was tested by ELISA for binding of IgG antibodies to solid-phase NS1 from different flaviviruses. Eighteen ZIKV-immune plasma samples, including 3 DENV-naive samples, 4 DENV-immune samples, and 11 samples with unknown prior DENV exposure history, were tested at a fixed dilution of 1:100 against NS1 antigen from ZIKV, DENV1-4, West Nile virus (WNV), and yellow fever virus (YFV) (Fig. 1*A*). All ZIKV-immune plasma samples recognized ZIKV NS1. Of note, the four plasma samples from DENV-immune donors all cross-reacted with DENV1-4 and WNV NS1, whereas the three samples from DENV-naive subjects showed weak to no cross-reactivity. Samples from patients with unknown DENV-immune status were for the most part specific for ZIKV NS1. Similarly, plasma samples from ZIKV-naive DENV-immune donors (including secondary DENV infections) were tested for IgG reactivity against flavivirus NS1 (Fig. 1*B*). Notably, a large fraction of these samples (i.e., 36–40%; cutoff, 1.5 OD) cross-reacted with ZIKV, WNV, and YFV NS1. None of the plasma samples from the healthy donors demonstrated reactivity against NS1 from different flaviviruses (Fig. 1*C*). Taken together, these results indicate that the cross-reactivity of anti-NS1 IgG antibodies induced by ZIKV and DENV infections represents an obstacle to the use of ELISA methods based solely on the binding of total IgG antibodies to solid-phase NS1 for development of flavivirus-specific serological assays. We therefore considered it likely that a competitive assay format, using labeled ZIKV-specific antibodies, would improve the specificity.

Analysis of Nicaraguan Samples. To resolve the issue of cross-reactive anti-NS1 antibodies, we took advantage of a human ZIKV-specific anti-NS1 mAb (ZKA35) we had previously generated and characterized from a recovered ZIKV-infected individual (10). We labeled this mAb with biotin and used it in an ELISA based on solid-phase ZIKV NS1. In this assay, plasma or serum samples are diluted (1:10) and incubated on ZIKV NS1-coated plates, followed by the addition of the biotinylated ZKA35 mAb, which detects the presence of ZIKV-specific serum antibodies capable of inhibiting its binding to ZIKV NS1. To evaluate the sensitivity and specificity of this NS1 BOB assay, we tested a large collection of longitudinal samples derived from multiple studies of Zika and dengue in Nicaragua (Table 1). A total of 112 and 146 samples from the latest times available from subjects diagnosed by RT-PCR for ZIKV or DENV infection, respectively, were used to perform a receiver operator characteristic (ROC) analysis to identify the cutoff giving optimum sensitivity and specificity in the NS1 BOB ELISA. The ROC analysis established 50% as the most effective cutoff value for ZKA35 binding inhibition (Fig. 2 *A–C*); that cutoff was therefore used throughout this study. Of the ZIKV panel, 37 and 38 subjects from the Nicaraguan Pediatric Dengue Cohort Study (PDCS) (13, 14) were defined as previously DENV-naive or DENV-immune, respectively, whereas DENV-immune status in 37 subjects from the Nicaraguan national surveillance system was not known. Testing of the samples collected at least 10 d after symptom onset in the NS1 BOB assay indicated that 100 of 112 samples scored positive (89.3%) (Fig. 2 *D–G*). In this dataset, we did not observe a significantly different rate of positivity in the NS1

Table 1. Characteristics of subjects included in the study

Population and location*	*N* subjects/ samples	Sex (% female)	Age mean (range)	Time† mean (range)
ZIKV				
NIC	112/215	65.1	18 (2–66)	32 (1–170)
BR	58/116	58.6	38 (3–67)	7 (1–53)
IT/UK	23/27	39.1	43 (18–68)	104 (3–753)
1° DENV				
NIC	59/65	54.2	8 (2–13)	44 (1–132)
IT	25/25	52.0	32 (6–58)	24 (2–200)
2° DENV				
NIC	87/99	48.2	10 (4–14)	31 (1–136)
IT	19/19	33.3	44 (15–68)	11 (3–30)
Acute DENV				
BR	82/82	50.0	30 (0–80)	4 (1–9)
WNV				
IT	49/49	12.2	60 (29–93)	35 (3–105)
YFV vaccine				
BR	14/14	30.7	18 (1–53)	—
IT	30/30	56.6	45 (12–76)	>1 y
CHIKV				
IT	10/10	50.0	42 (24–74)	23 (5–55)
Other disease				
BR	37/37	56.7	18 (0–74)	—
Healthy				
CH	116/116	—	—	NA
Pregnant				
UK	102/102	100.0	—	NA
Syst. illness‡				
UK	39/39	—	—	>14
Total	873/1,069	60.0	27 (0–93)	

NA, not applicable; —, not available.
*Location: BR, Brazil; CH, Switzerland; IT, Italy; NIC, Nicaragua; UK, United Kingdom.
†Time, days since symptom onset.
‡Symptomatic systemic illness.

MICROBIOLOGY

BOB assay in samples between DENV-naive and DENV-immune patients. The kinetics of the NS1 BOB assay results indicated that antibodies inhibiting the binding of the ZKA35 mAb probe persisted for more than 5 mo after ZIKV infection.

The specificity of the NS1 BOB assay in DENV-immune samples was further investigated by separately analyzing longitudinal samples from primary versus secondary DENV infections stratified by serotype. A total of 75 samples from 58 subjects diagnosed as primary DENV1, DENV2, or DENV3 infections by RT-PCR during 2010–2014 (before the introduction of ZIKV into Nicaragua) were analyzed; none of these samples scored positive (Fig. 2 *H*, *J*, and *K*). Similarly, 99 samples from 87 subjects diagnosed as secondary DENV1, DENV2, or DENV3 infections by RT-PCR from 2005 to 2016 were evaluated. In 17 of 86 secondary DENV infections, the NS1 BOB assay scored positive (Fig. 2 *I*–*K*). These results indicate that the ZIKV NS1 BOB assay is highly specific in patients with primary DENV and in the great majority (about four of five) of patients who experienced secondary DENV infections.

Analysis of Brazilian Samples. To further evaluate the performance and robustness of the NS1 BOB assay, we transferred the assay to the Flavivirus Laboratory at the Oswaldo Cruz Foundation (Fiocruz) in Rio de Janeiro and tested a panel of samples from 58 ZIKV-infected patients from Brazil collected between 2 and 53 d after symptom onset (Table 1). In most cases, multiple samples were collected from each patient. Twenty-nine of 31 samples (93.5%) collected more than 10 d after symptom onset scored positive in the NS1 BOB assay. In addition, 35 (41.2%) of 85 samples collected less than 10 d after symptom onset scored positive (Fig. 3 *A* and *B*), a result that might be related to an early production of NS1-reactive antibodies or to an inhibitory effect by circulating NS1. In parallel, we tested serum samples collected between 2002 and 2013 (before ZIKV introduction into Brazil) from 82 patients diagnosed with acute DENV infection by RT-PCR. Of these, only four samples scored positive (specificity of 95.1%). In addition, 62 samples collected from 2000 to 2014 from patients diagnosed with infection with measles virus, hepatitis B virus, human cytomegalovirus, rubella virus, hepatitis A, and hepatitis E viruses, as well as YFV vaccinees, were also tested using the NS1 BOB assay, and all scored negative. These results confirmed the high sensitivity and specificity of the NS1 BOB assay on a distinct group of patients and also demonstrate that the assay is sufficiently robust to be successfully established in different laboratories in multiple countries and in populations endemic to many flaviviruses.

Analysis of Samples from European Travelers and Residents. To assess the efficacy of the assay on samples from travelers returning from areas at risk for ZIKV infection, we tested plasma samples from 23 travelers in the United Kingdom and Italy who were confirmed by RT-PCR to have been infected with ZIKV; in most of these cases, the DENV immune status was not known. Of note, several plasma samples were collected more than 200 d after symptom onset (in one case, 753 d afterward). Twenty-one (95.5%) of 22 samples collected more than 10 d after symptom onset scored positive for ZIKV in the BOB ELISA (Fig. 4 *A* and *B*). To further assess the specificity of the ZIKV NS1 BOB assay, we tested a large set of samples obtained from patients infected with DENV, WNV, or chikungunya virus (CHIKV). Thirty-one (96.9%) of 32 samples from patients with WNV collected more than 10 d after symptom onset scored negative. Of note, the only positive was obtained from a sample collected in 2016. Two of 27 samples from patients with DENV collected more than 10 d after symptom onset scored positive, and similar to what was observed with Nicaraguan DENV samples, the two positive samples were derived from secondary DENV infections. In addition, none of the samples from patients with chikungunya or YFV vaccinees scored positive. We also tested a large number of plasma samples from Swiss blood donors (n = 116) collected between 2010 and 2016, as well as other samples derived from healthy pregnant women who underwent antenatal screening for HIV-1 in 2010 and 2015 (n = 102) and convalescent samples collected at least 14 d after illness from returning travelers who had been tested for leptospirosis (n = 39) as a result of undiagnosed systemic illness. Only two samples from this wider set of samples from healthy adults (blood donors and pregnant women) or UK individuals with undiagnosed systemic illness after travel (n = 257) scored positive; of note, these two samples were collected in 2015 and 2016, a period in which it is not possible to exclude a real exposure to ZIKV infection. Once more, the results obtained

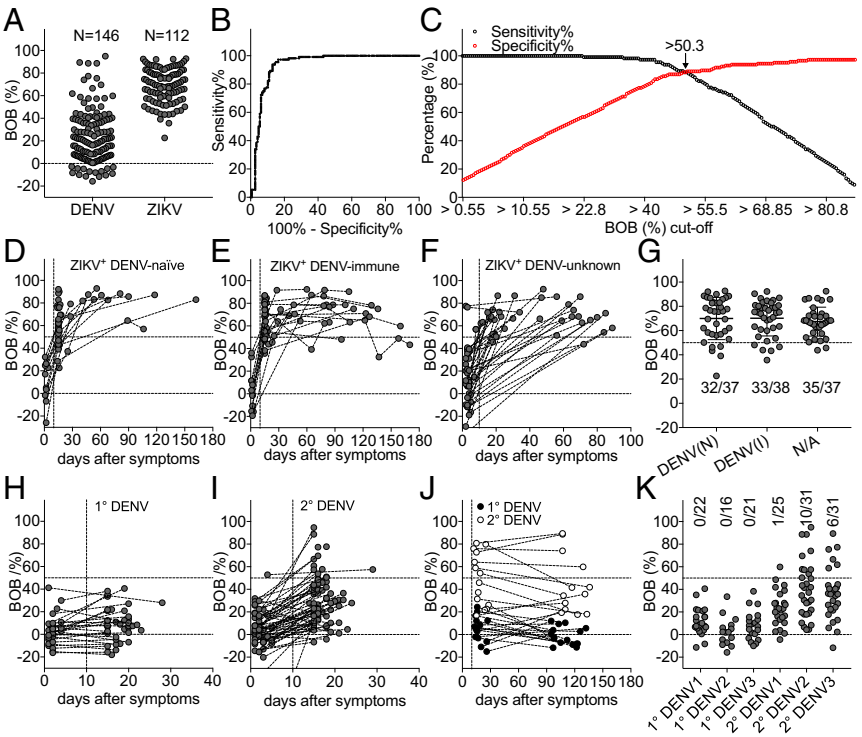

Fig. 2. Blockade of binding of ZIKV and DENV samples from Nicaraguan studies. (*A*) Percentage of ZKA35 mAb binding inhibition by ZIKV and DENV serum/plasma samples in the NS1 BOB assay. (*B*) ROC analysis performed on the results shown in *A*. (*C*) Sensitivity and specificity of various cutoff values for BOB inhibition, as based on the ROC analysis performed in *B*. (*D*–*K*) Percentage of blockade of binding, in relation to days after symptom onset, of serum/plasma samples from ZIKV-infected (*D*–*G*) or DENV-infected (*H*–*K*) patients. (*D*) DENV-naive, (*E*) DENV-immune, and (*F*) unknown DENV-immune status. (*G*) Shown is one value per subject selected as the latest point available >10 d postsymptom onset. DENV(I), DENV-immune; DENV(N), DENV-naive; N/A, prior DENV infection status not available. (*H*) Samples from primary DENV cases at acute and convalescent points. (*I*) Samples from secondary DENV cases at acute and convalescent points. (*J*) Samples from patients with dengue at convalescent and 3-mo points (empty symbols, secondary DENV infections; filled symbols, primary DENV infections). (*K*) One value per subject >10 d postsymptom onset from the different groups of dengue cases.

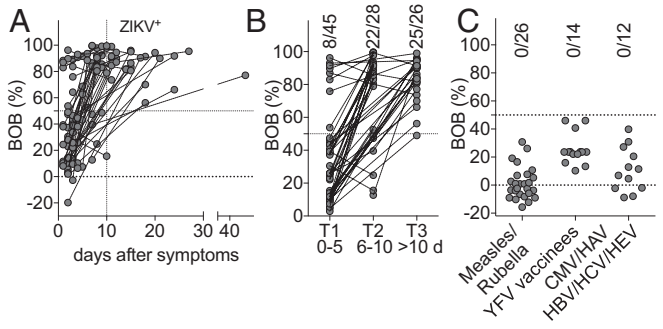

Fig. 3. NS1 BOB analysis of Brazilian samples. (*A*–*C*) Percentage of BOB in samples from (*A*) ZIKV-infected subjects versus the days postsymptom onset; (*B*) patients with Zika with several time points available, categorized as T1 (0–5 d), T2 (6–10 d), and T3 (>10 d); and (*C*) ZIKV-naive samples from subjects infected with measles virus, rubella, cytomegalovirus, hepatitis A virus, hepatitis B virus, and hepatitis C virus, as well as immunized with the YFV vaccine. Shown is the number of samples positive (i.e., BOB percentage >50%) out of the total.

confirmed and strengthened the high sensitivity and specificity of the NS1 BOB ELISA.

Sensitivity and Specificity of the NS1 BOB ELISA. To assess the degree of sensitivity and specificity of the NS1 BOB ELISA, we analyzed one sample from each patient (the latest available) and evaluated the frequency of patients developing an inhibitory antibody response. Because in the analysis of longitudinal samples shown in Figs. 2 and 3, we observed that the development of "inhibitory" antibodies required a time window of more than 10 days; here we excluded samples collected 10 d or less after symptom onset. In total, we analyzed samples from 158 patients with Zika from Nicaragua, Brazil, the United Kingdom, and Italy, and found that when the time cutoff was set at >10 d after symptom onset, the sensitivity was 91.8% (i.e., 145 of 158 positive; Fig. 5 *A* and *B*). When the cutoff was changed to ≥20 d after symptom onset, the sensitivity increased to 95.0%, and it remained at similar levels when the cutoff was further raised to ≥50 or ≥100 d after symptom onset (94.3% and 95.0%, respectively). Notably, in 96 of 158 samples, the percentage of BOB inhibition was above 70%, indicating that a high level of inhibitory antibodies is elicited by ZIKV infection. The analysis of the assay specificity was initially focused on samples from patients diagnosed for DENV infection between 2005 and 2014. Here we analyzed samples from 171 patients from Nicaragua and Italy >10 d postonset of symptoms and found a specificity of 88.9% (i.e., 152 of 171 samples scored negative). Of the 19 samples that scored positive, all were derived from patients with secondary DENV (mainly from secondary DENV2 and DENV3 infections). When the same analysis was restricted to samples collected from secondary DENV cases, we found that in 19 of 97 patients, the assay scored positive, yielding a specificity of 80.4%. Conversely, when the analysis was extended to all 257 patients infected (or vaccinated, in the case of YFV) with any flavivirus (i.e., DENV, WNV, or YFV), the specificity increased to 91.9% (i.e., 227 of 247 samples scored negative), and when it was further extended to all 540 control subjects used in this study, including blood donors, pregnant women, and samples from returned travelers, the specificity increased to 95.9% (i.e., 518 of 540 samples scored negative).

Discussion

The serological detection of ZIKV infections is challenging because of the high degree of cross-reactivity with other flaviviruses. Indeed, some have raised the possibility of considering ZIKV as a fifth member of the DENV serocomplex (15). A specific serological assay for detection of anti-ZIKV antibodies is urgently needed for surveillance of both Zika and dengue, age-stratified serosurveys, and studies investigating the association of ZIKV with congenital Zika syndrome and Guillain-Barré syndrome.

In this study, we initially found that measuring the binding of total IgG to solid-phase ZIKV NS1 did not enable significant discrimination of samples from patients with Zika and dengue, in that a large number of samples (i.e., ∼40%) from DENV-immune patients, collected during a period in which ZIKV exposure could be excluded, scored as positive in a direct ZIKV NS1-based ELISA. These results are consistent with our previous finding (10) that NS1-reactive monoclonal IgG antibodies isolated from patients with ZIKV and DENV can be cross-reactive. In particular, we found that more than 50% of the mAbs derived from patients with Zika who had been previously exposed to DENV were cross-reactive with DENV (in most cases with DENV1, DENV2, and DENV3, although this might be a reflection of the infection history of the donors), as predicted by the original antigenic sin theory in a recall response (16, 17). These results indicate that measuring the binding of IgG antibodies to ZIKV NS1 in an indirect ELISA format (18) results in low specificity.

Here, we evaluated an ELISA on the basis of the use of recombinant ZIKV NS1 protein as a solid-phase antigen and a labeled mAb (ZKA35) as probe, which was selected for its specificity to a site on ZIKV NS1 not targeted by cross-reactive antibodies. ZKA35 does not bind to heat-denatured NS1 (Fig. S1*A*), and thus likely targets a discontinuous or conformational epitope displayed on the surface of ZIKV NS1. Because ZKA35 mAb was isolated from an individual infected with ZIKV on a trip to Guatemala and El Salvador in November 2015 (19), it is plausible this antibody was elicited by NS1 antigen from an American ZIKV strain. However, it binds equally well to recombinant NS1 from a 2016 Suriname ZIKV strain, as well as from the prototype Ugandan strain MR766 (Fig. S1*B*). In addition, we found that ZKA35 reacts by flow cytometry with cells infected with the Asian ZIKV strain H/PF/2013 (Fig. S1*C*). Taken together, these data indicate that ZKA35 binds to a site on NS1 (defined as S2 in ref. 10) that is highly conserved in multiple ZIKV lineages, but that is not homologous in sequence and structure with the corresponding site on NS1 of other flaviviruses. Additional studies will be required to map in more detail the epitope of the ZKA35 mAb.

The NS1 BOB assay was developed as a BOB assay and measures the presence of serum antibodies capable of preventing the binding of the labeled mAb probe to solid-phase ZIKV NS1. Importantly, the NS1 BOB assay is robust and was easily transferred to multiple laboratories in Latin America and Europe. We have not determined the subclass of antibodies able to block the binding of the ZKA35 mAb. However, we cannot exclude that both IgM and IgG could be responsible for the observed inhibition, particularly in samples taken early after the onset of illness. Additional studies, such as IgG or IgM depletion, could be explored to assess whether

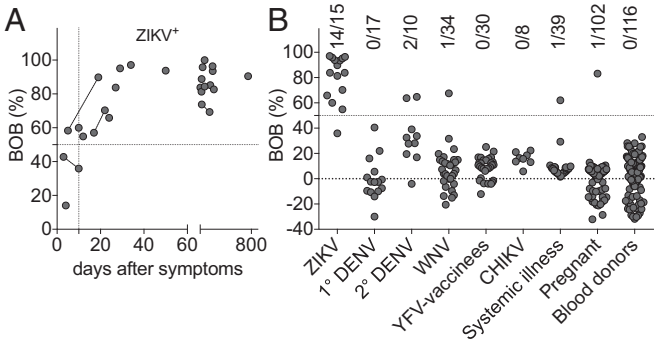

Fig. 4. NS1 blockade-of-binding analysis of European residents and returned travelers. (*A* and *B*) BOB values for samples collected in Italy, Switzerland, and the United Kingdom in (*A*) RT-PCR-confirmed ZIKV infections plotted over time. (*B*) Plotted are the BOB values in samples from ZIKV, primary and secondary DENV-, WNV-, and CHIKV-infected individuals and samples from United Kingdom-resident pregnant women from 2010 and 2015, a panel from UK individuals with symptomatic systemic illness and fever who were tested for leptospirosis (many of whom were returning travelers), and a panel of samples from healthy blood donors from Switzerland.

MICROBIOLOGY

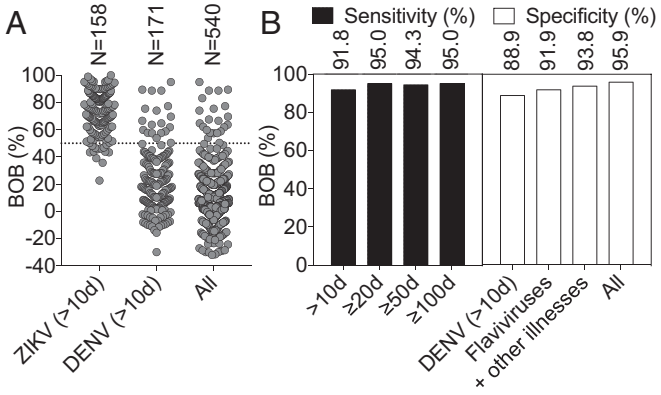

Fig. 5. Analysis of the sensitivity and specificity of the BOB NS1 ELISA. (*A*) The values of BOB inhibition in samples from subjects diagnosed with ZIKV or DENV infection by RT-PCR are shown. The DENV group consists of samples from primary and secondary DENV cases from Nicaragua and Italy. The control group identified as "all" comprises DENV samples as well as samples from patients infected (or vaccinated) with other flaviviruses (i.e., WNV and YFV) or with CHIKV, samples from patients with other illnesses and samples from healthy blood donors. (*B*) Sensitivity calculated on the basis of different time-point cutoff values, and specificity calculated using different control groups. The flaviviruses group comprises samples from DENV and WNV cases and YFV vaccinees. The control group identified as "+ other illnesses" comprises the samples from the flavivirus group, as well as samples with CHIKV and other illnesses (Brazilian samples collected before 2014).

IgM antibodies also contribute to the initial inhibition. Furthermore, in an acute setting, we cannot rule out that high levels of circulating ZIKV NS1 could also contribute to the inhibitory signal. Indeed, we have observed that in the NS1 BOB assay, the addition of exogenous soluble ZIKV NS1 (above 1 μg/mL) resulted in an inhibition of ZKA35 binding to solid-phase ZIKV NS1 (Fig. S2). This mechanism of inhibition could explain some of the positive results observed in samples from Brazilian patients collected less than 10 d after symptom onset (Fig. 3 *A* and *B*).

The sensitivity of the NS1 BOB assay was found to be ∼92%, and 10 of the 15 samples that scored as negative had a percentage of inhibition greater than 40%, suggesting sensitivity could be increased by further optimizing the assay conditions. Furthermore, 11 of the 15 negative ZIKV samples were collected less than 20 d postsymptom onset, and indeed, when only samples collected more than or equal to 20 d after symptom onset were considered, the sensitivity increased to 95%. Importantly, we found that antibodies blocking the binding of ZKA35 probe persisted over time without a trend for a clear decline, at least in the time window analyzed (i.e., up to 753 d after symptom onset).

The specificity of the NS1 BOB assay was found to range from 80.4% to 95.9%, depending on the control groups analyzed. Importantly, exposure to YFV vaccine or primary DENV infection did not result in any false-positive samples. Most of the samples scoring as false-positives (i.e., 19 of 22) were among the secondary DENV samples. Of note, the three positives from non-DENV samples were collected in 2015–2016 from patients who might have had a history of previous asymptomatic ZIKV infection. These results indicate that in a fraction (about 1 in 5) of patients with secondary DENV, cross-reactive antibodies targeting the ZKA35 site or neighboring sites on ZIKV NS1 are elicited by multiple exposures to DENV NS1 from different serotypes. It will be interesting to determine whether a given combination of sequential infections with different DENV serotypes is primarily responsible for eliciting cross-reactive antibodies able to block ZKA35 mAb binding to ZIKV NS1. ZIKV and DENV1-4 NS1 share homology in protein sequence of about 51–53%, whereas within DENV serotypes, DENV1 and DENV3 NS1 share homology at the amino acid level of about 81%, and DENV2 homology to DENV1 and DENV3 is considerably lower (i.e., 73% and 74%, respectively). DENV4 is equally distant from the other 3 serotypes (i.e., 71–75%). It will be interesting to understand whether sequential infection with more distant DENV serotypes is more likely to elicit antibodies able to inhibit ZKA35 binding to ZIKV NS1. Finally, it would be useful to isolate cross-reactive mAbs from patients with secondary DENV and map their fine specificity on ZIKV NS1. This could enable mutation of ZIKV NS1 to rationally knock out their epitopes with the goal of further increasing the specificity of the NS1 BOB assay.

The strengths of this study include the very well characterized samples from the cohort studies in Nicaragua that enabled distinction between DENV-naive and DENV-exposed ZIKV-infected patients, as well as samples from DENV infections stratified by serotype and immune status. Furthermore, acute, convalescent, and longitudinal samples from the same patients allowed careful kinetic analyses to be performed. This unique sample set was essential for providing time-matched comparisons of ZIKV infections versus DENV infections of different serotypes. One limitation of this study is that we could not test samples from subjects convalescent from DENV4 infections, as a result of the absence of DENV4 in our sample set.

Initial calculations estimate the cost per well in reagents and materials as approximately US$0.25; furthermore, the assay is based on an ELISA platform, which is widely available throughout the world. Thus, the NS1 BOB assay represents a broadly accessible, low-cost solution. Other important features of the NS1 BOB assay are its simplicity, its single-assay format, and its requirement for only one dilution of the samples compared with other tests requiring several dilutions and/or more complex and lengthy procedures (such as neutralization tests or Luminex-based assays).

Additional studies are ongoing to further simplify the ZIKV NS1 BOB assay protocol, including direct conjugation of the ZKA35 to peroxidase and the development of a lateral flow format. A similar BOB approach using other available mAbs reacting specifically with DENV1-4 NS1 antigen (such as DV54, described in ref. 12) could also be developed to identify previous DENV infections and to define the DENV-immune status of ZIKV-infected patients.

In conclusion, in response to the urgent need to develop a serological assay capable of distinguishing between ZIKV and DENV infections, we performed an extensive analysis of a large panel of well-characterized clinical samples derived both from travelers and from patients living in areas with a high level of exposure to ZIKV and endemic for other flaviviruses. Using this unique panel of samples from relevant populations, we have demonstrated the high sensitivity and specificity of a low-cost, accessible ZIKV NS1 serological assay. The NS1 BOB assay is thus a timely solution for Zika surveillance, seroprevalence studies, and intervention trials.

Materials and Methods

Study Design and Participants.

Samples from Nicaragua. A total of 75 children RT-PCR-positive for ZIKV who experienced signs and symptoms of Zika between January 18 and September 20, 2016, from the Nicaraguan Pediatric Dengue Cohort Study (PDCS) were included. The PDCS is a community-based prospective study of children 2–14 y of age that has been ongoing since August 2004 in Managua, Nicaragua (13). All Zika suspected cases were confirmed by RT-PCR in serum and/or urine, using triplex assays that simultaneously screen for DENV and CHIKV infections [ZCD assay (20), CDC Trioplex (21), or in some cases the CDC ZIKV monoplex assay (8), in parallel with a DENV-CHIKV multiplex assay (22)]. As a control group, a total of 146 patients with dengue confirmed by RT-PCR (23, 24) between 2005 and 2013 were included, of whom 115 (41 primary cases and 74 secondary cases) belonged to the PDCS and 31 (18 primary cases and 13 secondary cases) were participants in the Nicaraguan Hospital-based Dengue Hospital at the National Pediatric Reference Hospital in Nicaragua (25), which has been ongoing since 1998. The PDCS and the Dengue Hospital-based study were approved by the institutional review boards of the Nicaraguan Ministry of Health and the University of California, Berkeley. Parents or legal guardians of all subjects provided written informed consent, and subjects ≥6 y old provided assent. Full details are provided in the *SI Materials and Methods*.

Samples from Brazil. Samples from Brazilian patients used in this study were referred for routine diagnostics to the Flavivirus, Influenza, and Hepatitis reference laboratories at the Institute Oswaldo Cruz (IOC), Fiocruz-Rio de Janeiro (Table 1). ZIKV-positive patients ($n = 58$) were confirmed by RT-PCR, as previously described (8), and paired acute- and convalescent-phase serum

samples were collected during 2015 and 2016 (n = 116). Samples with confirmed DENV, measles, rubella, and other infections (PCR- and/or IgM-positive sera) or YFV vaccination were collected in or before 2013 to exclude ZIKV exposure, which is estimated to have arrived in Rio de Janeiro in January 2015. These samples belong to a previously gathered collection from the Laboratory of Flavivirus, IOC/Fiocruz approved by resolution number CSN196/96 from the Oswaldo Cruz Foundation Ethical Committee in Research (CEP 274/05), Ministry of Health, Brazil. Samples were received for routine diagnostics as part of the reference work of the laboratory. The ethics approval from the committee includes further studies using these samples for improvement of the diagnostic assays without needing informed consent of these patients.

Samples from Italy. The diagnostic assessment for DENV, ZIKV, CHIKV, WNV and YFV included the following: detection of anti-DENV IgM and IgG antibodies in serum samples, detection of ZIKV IgM and IgG antibodies, identification of CHIKV IgM and IgG using specific immunofluorescence tests, detection of WNV IgM and IgG using specific tests, and detection of YFV IgM and IgG with anti-YFV immunofluorescence assays (IFA). Serology results were confirmed by specific virus neutralization assays (26). Detection of DENV RNA, ZIKV RNA, and WNV RNA in plasma and urine samples was performed using a pan-flavivirus heminested RT-PCR assay targeting a conserved region of the NS5 gene (27), as well as virus-specific real-time RT-PCR tests. This retrospective analysis was performed according to guidelines of the institutional review board of the Fondazione IRCCS Policlinico San Matteo on the use of biologic specimens for scientific purpose, in keeping with Italian law (art.13 D.Lgs 196/2003). Informed consent was not necessary because patients with suspected arbovirus infections were included in a regional diagnostic protocol. Full details are provided in *SI Materials and Methods*.

Samples from the United Kingdom. Samples from UK patients used in this study included the following: a cohort of Zika-positive returning travelers undergoing long-term follow up, anonymized residual samples from antenatal screening of healthy pregnant women, and residual convalescent sera from individuals with systemic illness who tested negative for leptospirosis. Postinfection sera from Zika-infected UK blood donors were collected with full informed consent in accordance with and as required by the National Health Service Blood Transfusion Service, England. Sera remaining after antenatal screening of healthy pregnant women or testing of returning UK travelers for leptospirosis were used anonymously, in accordance with Royal College Pathologists guidelines.

Production and Labeling of the ZKA35 mAb Probe. The V_H and V_L sequences of ZKA35 human mAb were cloned into human Igγ1 and Igκ expression vectors (28), and recombinant ZKA35 mAb was produced by transient transfection of EXPI293 cells (Invitrogen), essentially as previously described (28). Full details are provided in *SI Materials and Methods*.

NS1 Blockade-of-Binding Assay. Polystyrene plates were coated overnight with 1 μg/mL ZIKV and blocked for 1 h with PBS containing 1% BSA. Plasma or serum (1:10 dilution) were added to NS1-coated ELISA plates. After 1 h, an equal volume of biotinylated anti-NS1 ZKA35 was added, and the mixture was incubated at room temperature for 15 min. Plates were washed, and alkaline-phosphatase-conjugated streptavidin was added for 30 min. Plates were washed again, and the substrate was added for 30–60 min. The percentage of inhibition was calculated as follow: [1 − ([OD sample − OD neg ctr]/[OD pos ctr − OD neg ctr])] × 100. Full details are provided in *SI Materials and Methods*.

Statistical Analysis. ROC analysis was performed using GraphPad Prism 7 (GraphPad Software Inc.). Full details are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank past and present members of the study team based at the Centro de Salud Sócrates Flores Vivas, Hospital Infantil Manuel de Jesús Rivera, the National Virology Laboratory in the Centro Nacional de Diagnóstico y Referencia, and the Sustainable Sciences Institute in Nicaragua for their dedication and high-quality work, as well as the children who participated in the studies and their families. We thank the Flavivirus Laboratory, the Influenza Laboratory, and the Viral Hepatitis Laboratory from Fiocruz and the Evandro Chagas National Institute of Infectious diseases for providing the samples from Brazil. We thank the staff of the Rare and Imported Disease Laboratory at Public Health England (PHE) in Porton Down, the staff of virus and bacterial reference divisions at PHE Colindale for technical assistance, and particularly Dr. Jennifer Tossiwil, Dr. Colin Brown, Dr. Tim Brooks, and Dr. Emma Aarons for their assistance. Partial funding support was provided by National Institutes of Health Grants P01AI106695 (to E.H.), U19AI118610 (to E.H.), and R01AI099631 (to A.B.). R.M.-C.'s work is supported by the National Institute for Health Research (Health Protection Research Unit) in Emerging and Zoonotic Infections at the University of Liverpool in partnership with PHE and Liverpool School of Tropical Medicine. This work was also supported by Medical Research Council "Rapid response to Zika virus" Grant MC_PC_15093.

1. Oliveira Melo AS, et al. (2016) Zika virus intrauterine infection causes fetal brain abnormality and microcephaly: Tip of the iceberg? *Ultrasound Obstet Gynecol* 47:6–7.
2. Moore CA, et al. (2017) Characterizing the pattern of anomalies in congenital Zika syndrome for pediatric clinicians. *JAMA Pediatr* 171:288–295.
3. Parra B, et al. (2016) Guillain–Barré syndrome associated with Zika virus infection in Colombia. *N Engl J Med* 20;375:1513–1523.
4. Muller DA, Young PR (2013) The flavivirus NS1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. *Antiviral Res* 98:192–208.
5. Katzelnick LC, Coloma J, Harris E (2017) Dengue: Knowledge gaps, unmet needs, and research priorities. *Lancet Infect Dis* 17:e88–e100.
6. Bingham AM, et al. (2016) Comparison of test results for Zika virus RNA in urine, serum, and saliva specimens from persons with travel-associated Zika virus disease - Florida, 2016. *MMWR Morb Mortal Wkly Rep* 65:475–478.
7. Gourinat A-C, O'Connor O, Calvez E, Goarant C, Dupont-Rouzeyrol M (2015) Detection of Zika virus in urine. *Emerg Infect Dis* 21:84–86.
8. Lanciotti RS, et al. (2008) Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. *Emerg Infect Dis* 14:1232–1239.
9. Priyamvada L, et al. (2016) Human antibody responses after dengue virus infection are highly cross-reactive to Zika virus. *Proc Natl Acad Sci USA* 113:7852–7857.
10. Stettler K, et al. (2016) Specificity, cross-reactivity, and function of antibodies elicited by Zika virus infection. *Science* 353:823–826.
11. Harrison SC (2016) Immunogenic cross-talk between dengue and Zika viruses. *Nat Immunol* 17:1010–1012.
12. Beltramello M, et al. (2010) The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. *Cell Host Microbe* 8:271–283.
13. Kuan G, et al. (2009) The Nicaraguan pediatric dengue cohort study: Study design, methods, use of information technology, and extension to other infectious diseases. *Am J Epidemiol* 170:120–129.
14. Gordon A, et al. (2013) The Nicaraguan pediatric dengue cohort study: Incidence of inapparent and symptomatic dengue virus infections, 2004-2010. *PLoS Negl Trop Dis* 7:e2462.
15. Dejnirattisai W, et al. (2016) Dengue virus sero-cross-reactivity drives antibody-dependent enhancement of infection with zika virus. *Nat Immunol* 17:1102–1108.
16. Halstead SB, Rojanasuphot S, Sangkawibha N (1983) Original antigenic sin in dengue. *Am J Trop Med Hyg* 32:154–156.
17. Midgley CM, et al. (2011) An in-depth analysis of oringinal antigenic sin in dengue virus infection. *J Virol* 85:410–421.
18. Steinhagen K, et al. (2016) Serodiagnosis of Zika virus (ZIKV) infections by a novel NS1-based ELISA devoid of cross-reactivity with dengue virus antibodies: A multicohort study of assay performance, 2015 to 2016. *Euro Surveill* 21:30426.
19. Ginier M, Neumayr A, Günther S, Schmidt-Chanasit J, Blum J (2016) Zika without symptoms in returning travellers: What are the implications? *Travel Med Infect Dis* 14: 16–20.
20. Waggoner JJ, et al. (2016) Single-reaction multiplex reverse transcription PCR for detection of Zika, chikungunya, and dengue viruses. *Emerg Infect Dis* 22:1295–1297.
21. US Food and Drug Administration (2016) Zika virus emergency use authorization. Silver Spring, MD: US Department of Health and Human Services; 2016.
22. Waggoner JJ, et al. (2016) Clinical evaluation of a single-reaction real-time RT-PCR for pan-dengue and chikungunya virus detection. *J Clin Virol* 78:57–61.
23. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *J Clin Microbiol* 30:545–551.
24. Harris E, et al. (1998) Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *J Clin Microbiol* 36:2634–2639.
25. Narvaez F, et al. (2011) Evaluation of the traditional and revised WHO classifications of Dengue disease severity. *PLoS Negl Trop Dis* 5:e1397.
26. Timiryasova TM, et al. (2013) Optimization and validation of a plaque reduction neutralization test for the detection of neutralizing antibodies to four serotypes of dengue virus used in support of dengue vaccine development. *Am J Trop Med Hyg* 88: 962–970.
27. Scaramozzino N, et al. (2001) Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol* 39:1922–1927.
28. Tiller T, et al. (2008) Efficient generation of monoclonal antibodies from single human B cells by single cell RT-PCR and expression vector cloning. *J Immunol Methods* 329: 112–124.
29. Fernández RJ, Vázquez S (1990) Serological diagnosis of dengue by an ELISA inhibition method (EIM). *Mem Inst Oswaldo Cruz* 85:347–351.
30. Balmaseda A, et al. (2006) High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. *Trop Med Int Health* 11: 935–942.
31. Gutiérrez G, et al. (2013) Evaluation of the diagnostic utility of the traditional and revised WHO dengue case definitions. *PLoS Negl Trop Dis* 7:e2385.
32. Huhtamo E, et al. (2010) Early diagnosis of dengue in travelers: Comparison of a novel real-time RT-PCR, NS1 antigen detection and serology. *J Clin Virol* 47:49–53.
33. Pastorino B, et al. (2005) Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African Chikungunya viruses. *J Virol Methods* 124:65–71.
34. Linke S, Ellerbrok H, Niedrig M, Nitsche A, Pauli G (2007) Detection of West Nile virus lineages 1 and 2 by real-time PCR. *J Virol Methods* 146:355–358.

Supporting Information

Balmaseda et al. 10.1073/pnas.1704984114

SI Materials and Methods

Study Design and Participants.

Samples from Nicaragua. A total of 75 children RT-PCR-positive for ZIKV who experienced signs and symptoms of Zika between January 18 and September 20, 2016, from the Nicaraguan PDCS were included. The PDCS is a community-based prospective study of children 2–14 y of age that has been ongoing since August 2004 in Managua, Nicaragua (13). Participants present at the first sign of illness to the Health Center Sócrates Flores Vivas, and study physicians use standardized forms to collect clinical data. Subjects are followed daily during the acute phase of illness. In 2014, CHIKV was added to the PDCS, and in January 2016, ZIKV screening was initiated of participants meeting the clinical definition of dengue and/or chikungunya or presenting with undifferentiated fever. In February, the case definition was expanded to include those presenting with signs and symptoms of Zika, but without fever. Acute and convalescent (~14 d after onset of symptoms) blood samples are drawn for dengue, chikungunya, and Zika diagnostic testing. If confirmed ZIKV-positive subjects presented subsequently with another febrile episode, a blood sample was collected for clinical management and stored; these samples were also included in the current study. In the PDCS, a healthy blood sample is collected annually from participants; anti-DENV antibody titers are measured in paired annual samples using an inhibition ELISA (29, 30), and infections are defined by seroconversion or a fourfold or greater rise in anti-DENV titers. In this study, confirmed ZIKV cases were classified as DENV-naive if they entered the cohort study with no detectable anti-DENV antibodies (as measured by inhibition ELISA) and had no documented DENV infections (symptomatic or inapparent) during their time in the cohort, or were classified as DENV-immune if they either entered the cohort with detectable anti-DENV inhibition ELISA antibodies or entered the cohort study with no detectable anti-DENV antibodies and had one or more documented DENV infections during their time in the cohort. We also included 37 patients with Zika from the Nicaraguan National Surveillance System who become ill between January 26 and May 11, 2016, and were confirmed by RT-PCR by the National Virology Laboratory of the Ministry of Health. Late convalescent samples were collected weeks to months after onset of symptoms and were included in this study as well. All suspected Zika cases were confirmed by RT-PCR in serum and/or urine, using triplex assays that simultaneously screen for DENV and CHIKV infections [ZCD assay (20), CDC Trioplex (21)], or in some cases the CDC ZIKV monoplex assay (8) in parallel with a DENV-CHIKV multiplex assay (22).

As a control group, a total of 146 patients with dengue confirmed by RT-PCR (23, 24) between 2005 and 2013 were included, of which 115 (41 primary cases and 74 secondary cases) belonged to the PDCS and 31 (18 primary cases and 13 secondary cases) were participants in the Nicaraguan Hospital-based Dengue Hospital at the National Pediatric Reference Hospital in Nicaragua (25), which has been ongoing since 1998. Primary DENV infection was defined by an antibody titer by inhibition ELISA of <2,560 in convalescent-phase samples (31). During the peak dengue season (August–January), children between 6 mo and 14 y of age with suspected dengue disease who present to the hospital within 7 d of symptom onset are eligible to participate; in 2014, chikungunya was added, and in 2016, Zika was included in the study. Blood samples are collected at enrollment, during the acute phase, and 2–4 wk after symptom onset (convalescent phase) for clinical laboratory tests and dengue diagnostic tests. Subjects also can consent to participate in a longitudinal arm of the study, in which a medical examination is conducted and blood samples are collected 3, 6, 12, and 18 mo postillness. Samples from acute, convalescent, and 3-mo visits were included in this study. The PDCS and the Dengue Hospital-based study were approved by the institutional review boards of the Nicaraguan Ministry of Health and the University of California, Berkeley. Parents or legal guardians of all subjects provided written informed consent, and subjects ≥6 y old provided assent.

Samples from Italy. The diagnostic assessment for DENV, ZIKV, CHIKV, WNV, and YFV included the following: detection of anti-DENV IgM and IgG antibodies in serum samples (using dengue virus IgM Capture DxSelect and dengue virus IgG DxSelect, Focus Diagnostics, United States), detection of ZIKV IgM and IgG antibodies [anti-Zika virus ELISA (IgM) and anti-Zika virus ELISA (IgG); Euroimmun], identification of CHIKV IgM and IgG using specific immunofluorescence tests [anti-chikungunya virus IFA (IgG), anti-chikungunya virus IFA (IgM); Euroimmun], detection of WNV IgM and IgG using specific tests [NovaLisaTM Dengue IgM and IgG ELISAs (NovaTec Immunodiagnostic GmbH); and WNV IgM Capture DxSelect and WNV IgG DxSelect (Focus Diagnostics)], and detection of YFV IgM and IgG with anti-yellow fever virus IFA (IgG) and anti-yellow fever virus IFA (IgM; Euroimmun). Serology results were confirmed by specific viruses neutralization assays (26). Detection of DENV RNA, ZIKV RNA, and WNV RNA in plasma and urine samples was performed using a pan-flavivirus heminested RT-PCR assay targeting a conserved region of the NS5 gene (27), as well as virus-specific real-time RT-PCR tests, targeting a conserved region in the 3′ untranslated region of DENV1–DENV4 (32), a portion of the envelope protein gene of ZIKV (8), a portion of the E1 structural protein region of CHIKV (33), and a conserved region of the 5′-UTR and a part of the capsid gene of WNV lineages 1 and 2 (34). Sequencing was performed on positive pan-flavivirus amplicons to type DENV and WNV viruses. This retrospective analysis was performed according to guidelines of the institutional review board of the Fondazione IRCCS Policlinico San Matteo on the use of biologic specimens for scientific purpose in keeping with Italian law (art.13 D.Lgs 196/2003). The personnel of the Molecular Virology Unit participated in the collection of these samples, which were anonymized on collection. The local ethics committee consent was not required because according to a Regional Surveillance and Preparedness Plan (DGR 12591, December 27, 2012), diagnostic detection of arbovirus infections in the Lombardy Region was centralized at the Regional Reference Laboratory (Molecular Virology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia). Informed consent was not necessary because patients with suspected arbovirus infections were included in a regional diagnostic protocol. Prospective samples (serum, cerebrospinal fluid, and urine) were collected by clinicians and handled by Molecular Virology Unit personnel; data were analyzed anonymously according to a Regional Surveillance and Preparedness Plan (DGR 12591, December 27, 2012).

Production and Labeling of the ZKA35 mAb Probe. The V_H and V_L sequences of ZKA35 human mAb were cloned into human Igγ1, Igκ expression vectors (kindly provided by Michel Nussenzweig, Rockefeller University), essentially as previously described (28). Recombinant ZKA35 mAb was produced by transient transfection of EXPI293 cells (Invitrogen) purified by protein A chromatography (GE Healthcare), and desalted against PBS. ZKA35 IgG1 mAb was biotinylated using the EZ-Link NHS-PEG solid phase biotinylation kit (Pierce). Labeled ZKA35 was tested for

binding to ZIKV NS1 to determine the optimal concentration to achieve 70% maximal binding.

NS1 BOB Assay. Polystyrene plates (Nunc MaxiSorp) were coated overnight with 1 μg/mL ZIKV NS1 (MR766 strain; Meridian or Native Antigen Company, Inc.) in coating buffer (PBS) at 4 °C. Plates were blocked for 1 h with PBS containing 1% BSA. Plasma or serum (1:10 dilution in PBS/1% BSA) were added to NS1-coated ELISA plates. After 1 h, an equal volume of biotinylated anti-NS1 ZKA35 at 20 ng/mL was added, and the mixture was incubated at room temperature for 15 min (final dilution of the plasma in the mixture, 1:20). Plates were washed with PBS-T (PBS plus 0.05% Tween 20), and alkaline-phosphatase-conjugated streptavidin was added for 30 min. Plates were washed again with PBS-T, and the substrate (p-NPP, Sigma) was added for 30–60 min. Plates were read in an ELISA reader at 405 nm. The percentage of inhibition was calculated as follows: [1 − ([OD sample − OD neg ctr]/[OD pos ctr − OD neg ctr])] × 100.

Statistical Analysis. Sensitivity was calculated as the proportion of samples from ZIKV-infected individual identified as positive by the assay (percentage of inhibition >50%), using one sample per individual (the latest time available, and only for samples collected >10 d after symptom onset). Specificity was calculated as the proportion of negative test results obtained among the different control groups used.

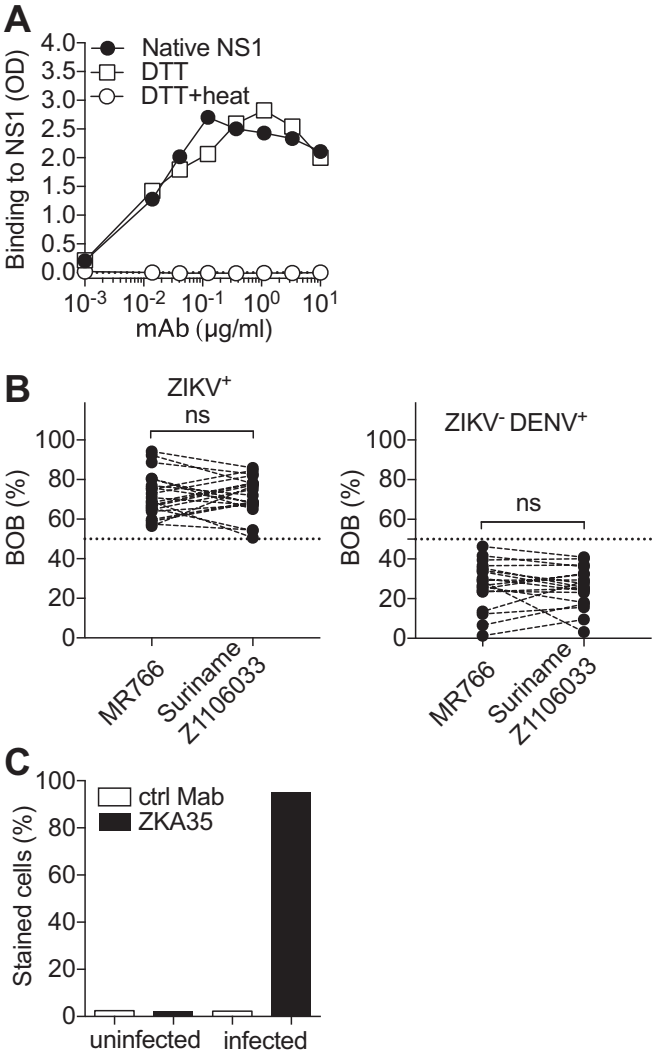

Fig. S1. ZKA35 binds to a conformational and highly conserved site on ZIKV NS1. (*A*) Binding of ZKA35 mAb to native, DTT-treated, or heated and DTT-treated NS1 as determined by ELISA. (*B*) BOB assay of ZIKV and DENV plasma samples from Nicaraguan studies, using NS1 from Uganda (MR766) and Suriname (Z1106033; both from Native Antigen Company, Inc.). Plotted is the percentage of ZKA35 mAb binding inhibition by 21 ZIKV-positive (*Left*) and 20 DENV-positive, ZIKV-negative (*Right*) plasma samples tested in parallel, using NS1 from MR766 and Suriname. The DENV-positive samples included DENV1 (n = 9) and DENV2 (n = 11) infections, as well as primary (n = 4) and secondary (n = 16) infections. Statistical analysis performed using Wilcoxon matched-pairs signed rank test: ns, not significant. The average inhibition in ZIKV-positive patients was 71.7 versus 71.6, and in DENV-positive, ZIKV-negative patients, it was 23.5 versus 24.3 for Uganda and Suriname NS1, respectively. (*C*) Binding of ZKA35 mAb to Vero cells infected with H/PF/2013 ZIKV strain. ZKA35 and a control mAb (MPE8 directed to RSV F protein) were tested at 0.3 μg/mL, using mock- or ZIKV-infected cells 2 d postinfection. Cells were permeabilized with 0.5% saponin, and binding was determined using an anti-human IgG Alexa488-conjugated antibody in the presence of the Draq5 counterstain. Fluorescence was read using a high-throughput fluorometric microvolume assay technology (Mirrorball, TPP Labtech).

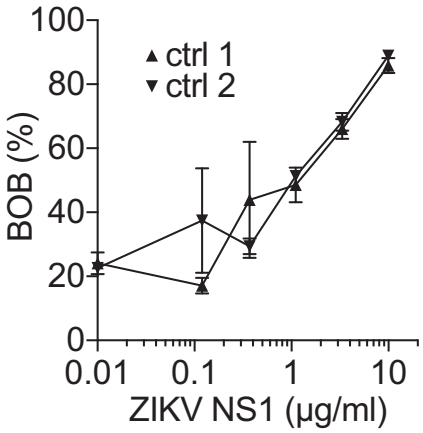

Fig. S2. Soluble NS1 inhibits ZKA35 binding to solid-phase ZIKV NS1. Plotted is the percentage of ZKA35 mAb binding inhibition by two plasma samples from Swiss blood donor controls (ZIKV- and DENV-negative) that were spiked with increasing amounts of ZIKV NS1. The addition of exogenous NS1 to negative control plasma samples resulted in ZKA35 mAb binding inhibition when the concentration of NS1 exceeded 1 μg/mL.