Rapid Antigen Tests for Dengue Virus Serotypes and Zika Virus in Patient Serum

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Abstract

The recent Zika virus (ZIKV) outbreak demonstrates that cost-effective clinical diagnostics are urgently needed to detect and distinguish viral infections to improve patient care. Unlike dengue virus (DENV), ZIKV infections during pregnancy correlate with severe birth defects, including microcephaly and neurological disorders. Because ZIKV and DENV are related flaviviruses, their homologous proteins and nucleic acids can cause cross reactions and false positive results in molecular, antigenic, and serologic diagnostics. We report here the characterization of monoclonal antibody pairs that have been translated into rapid immunochromatography tests to specifically detect the viral nonstructural 1 (NS1) protein antigen and distinguish the four DENV serotypes and ZIKV without cross reaction. To complement visual test analysis and remove user subjectivity in reading test results, image processing and data analysis were used for data capture and test result quantification. Using a 30 μl serum sample, the sensitivity and specificity values of the DENV serotypes 1–4 tests and the pan DENV test, which detects all four dengue serotypes, ranged from 0.76 to 1.00. Sensitivity/specificity for the ZIKV rapid test was 0.81/0.86 using a 150 μl serum input. Serum ZIKV NS1 protein concentrations were approximately tenfold lower than corresponding DENV NS1 concentrations in infected patients; moreover, ZIKV NS1 protein was not detected in PCR-positive patient urine samples. Our rapid immunochromatography approach and reagents have immediate application in differential clinical diagnosis of acute ZIKV and DENV cases, and the platform can be applied toward developing rapid antigen diagnostics for emerging viruses.

One Sentence Summary

A low-cost equipment-free rapid antigen test distinguishes dengue virus serotypes and Zika virus in patient sera without detectable cross reactivity.

Introduction

Genus *Aedes* mosquitoes transmit Dengue virus (DENV) and Zika virus (ZIKV), which are related flaviviruses and important global pathogens that inflict a heavy burden on public health systems. Dengue has a wide distribution, with more than a quarter of the world's population at risk, and hundreds of millions of new infections annually. There are four DENV serotypes, and infection with one of the four DENV serotypes fails to provide long-lasting immunity against the remaining three viral serotypes. Some reports suggest that disease severity varies among DENV serotypes (1). ZIKV gained global attention in 2015 when thousands of clinical cases appeared suddenly in northern Brazil, with accompanying reports of devastating congenital defects, including microcephaly and Guillain-Barre syndrome. The World Health Organization subsequently declared ZIKV a global public health emergency.

Cost-effective diagnostics are urgently needed to detect and distinguish DENV and ZIKV, as well as other pathogenic viruses. The flavivirus NS1 protein is a useful infection marker because of its release from infected cells into the bloodstream and its accumulation in dengue patients at concentrations up to 50 μg/ml (2). A number of commercial DENV NS1 rapid tests have been reported and compared (3–9). However, these studies were performed prior to the ZIKV epidemic, and a recent publication confirms DENV-ZIKV NS1 cross-reactivity using a commercial rapid test (10). In addition, none of the commercial rapid tests distinguishes the DENV serotypes.

Combining NS1 with immunoglobulin M (IgM), and immunoglobulin G (IgG) detection in a dual test improves sensitivity and specificity for DENV compared to NS1 alone (3, 4, 8). However, the primary antigen for IgG and IgM during flavivirus infections is the viral envelope (E) protein, and the similarity of the flavivirus envelope proteins fuels additional problems with cross reactivity and false positive test results (11). There are currently no approved or widely used vaccines to prevent ZIKV or DENV infections, nor low-cost rapid antigen-based diagnostics demonstrated to identify ZIKV infections without cross reactive interference of related dengue viruses. Here, we describe viral NS1 antigen-based rapid tests that use monoclonal antibody pairs to detect and distinguish the four DENV serotypes, as well as ZIKV, without cross reactivity.

Results

Approach for developing a rapid diagnostics platform to detect viral antigens

The stepwise approaches used to develop antigen-based rapid diagnostics are shown in fig. S1 and table S1. The percentages of amino acid homology and identity among flavivirus NS1 proteins are high (table S2); therefore, we reasoned that extensive and strategic screening would be required to identify monoclonal antibodies that detect and distinguish the viruses in a rapid diagnostic test. We tested several commercial anti-dengue NS1

antibodies available from different vendors, but found that native NS1 protein was not recognized or that there was clearly identifiable cross reactive binding among the DENV serotype NS1 proteins. We therefore generated and characterized anti-NS1 antibodies (fig. S1, table S1). Groups of mice were injected separately with DENV serotypes 1–4 recombinant NS1, or with ZIKV recombinant NS1. B cells from the spleen or lymph nodes were fused with mouse myeloma cells to generate hybridomas. Initial hybridoma screening (209 DENV hybridomas and 104 ZIKV hybridomas) was performed by enzyme-linked immunosorbent assay (ELISA), using individual recombinant NS1 protein as antigen (step 1, table S1). By screening the hybridoma supernatants against individual DENV serotypes 1–4 or ZIKV NS1 proteins, the relative ELISA values provided an initial evaluation of differential binding properties for each antibody.

Because of the similarities among the DENV and ZIKV NS1 proteins (table S2), each group of mice immunized with a single purified recombinant NS1 protein yielded a pool of antibodies showing both selective binding to a single DENV serotype or ZIKV NS1, as well as cross-reactive DENV and ZIKV antibodies (fig. S2). ZIKV NS1 hybridomas produced from lymph node tissue represented a higher proportion of clones showing minimal cross reactivity with dengue NS1 or other flavivirus NS1 proteins, as compared to spleen cell hybridomas (fig. S2). Using the relative ELISA values, 11 DENV monoclonal antibodies (mAb) and 10 ZIKV mAb were selected for further analysis. In step 2 (fig. S1; table S1), hybridoma supernatants were used to stain permeabilized Vero cells that had been infected with known ZIKV or DENV viral serotypes. Flow cytometry analysis demonstrated that the monoclonal antibodies recognized native NS1 protein expressed by virus-infected cells, and provided a quantitative analysis of cross reactive binding when ZIKV antibodies were used to stain DENV-infected cells and vice versa. The 11 DENV mAb and 10 ZIKV mAb recognized NS1 protein present in the virus-infected cells; therefore, the hybridomas were expanded and the mAb isotypes were defined in preparation for affinity chromatography purification.

The purified antibodies were tested in immunochromatography pairs (fig. S1; table S1, step 3), with one antibody conjugated to gold nanoparticles and one antibody adsorbed to nitrocellulose membrane. The 11 DENV hybridomas were tested in a matrix for interactions with DENV serotype 1–4 NS1, ZIKV NS1, or without added antigen as a control. The 10 ZIKV mAb were also tested in a matrix using ZIKV NS1, a mixture of the four DENV serotype NS1 proteins, or no antigen as a control. We tested 726 DENV combinations ($11 \times 11 \times 6 = 726$) and 300 ZIKV combinations ($10 \times 10 \times 3 = 300$). Testing throughput was increased by using the half-strip dipstick format (12), where dipsticks are run in rapid format (approximately 20 minutes, depending on humidity conditions) by placing them in microcentrifuge tubes containing small volume suspensions of conjugated nanoparticles and sample without need for sample paper pads and conjugate paper pads that are characteristic of lateral flow chromatography. The DENV and ZIKV matrices and the immunochromatography results are shown in table S3. Eight DENV mAb and two ZIKV mAb were ultimately incorporated into the rapid tests used to analyze patient samples. The ten mAb names, their relative NS1 recognition values in initial screening, and final application in the rapid tests are presented in Fig. 1A and table S4.

Linear epitope mapping; mechanisms of specific mAb-NS1 interactions

To begin to define mechanisms of specific mAb-NS1 interactions, we performed linear peptide epitope mapping (fig. S1; table S1, step 4). Libraries of tiled DENV NS1 peptides were spotted onto nitrocellulose membranes and incubated with antibody. After washing, positive signals were detected using an anti-mouse IgG antibody coupled to horseradish peroxidase for signal development. In a second approach, tiled peptides were synthesized on glass slides and incubated with each of the antibodies. Positive signals were detected by immunofluorescence microscopy and scored. The epitope mapping data are summarized in Fig. 1. mAb 7724.323 ("323") fits the definition of a pan-DENV NS1 antibody because it recognizes all four serotype NS1 proteins in the epitope 109–124 amino acid region. Six antibodies used in the rapid tests (using suffix nomenclature only: 55; 323; 1; 130; 110; 271) recognize the amino acid 109–124 epitope of the "wing" domain (amino acids 30–180) (13, 14). However, with the exception of a few pairs with mAb 243, these six antibodies do not recognize the ZIKV NS1 protein (Fig. 1B; table S3). The epitope mapping helped to explain the observed antibody specificity in the rapid tests. The shared DENV 1–4 NS1 region D/ ELKYSWKTWG (amino acids 110–119) (15) is not conserved in ZIKV NS1; rather the comparable ZIKV NS1 region has a distinct amino acid sequence (Fig. 1B), explaining how DENV NS1 mAbs, represented by the members of the 1/55/110/130/271/323 mAb group, do not cross react with the ZIKV NS1 protein. mAb 243, which showed high specificity for DENV serotype 2 (tables S3 and S4), recognized peptide 313–330 in DENV2 NS1(Fig. 1B). The screening approaches, combined with the epitope mapping, contributed to selecting antibodies representing positional epitope diversity; that is, epitopes in both the wing/wing-connector/finger and β-ladder domains (Fig. 1).

Matrix-based screening (table S3) revealed that the following pairs (nanoparticle/membrane) had optimal rapid test specificity: DENV1: 912/271; DENV2: 243/1; DENV3: 411/55; DENV4: 626/55. In addition, the 912 and 243 antibodies displayed excellent single serotype specificity for the DENV1 and DENV2 proteins, respectively. These antibodies might be predicted to work as 912/912 and 243/243 homo-pairs in detecting DENV1 and DENV2 NS1 dimers. However, experimental results showed that the limits of NS1 detection was improved by using hetero-pairs without sacrificing specificity; therefore, 912 was paired with 271, and 243 with 1. For ZIKV NS1 detection we used the 130/110 mAb pair, where both antibodies arose from the lymph node tissue approach (fig. S2). The 411, 55, and 626 antibodies did not display the single serotype specificity observed with 912 and 243 (table S4); however, when used as pairs in the rapid tests, 411/55 (DENV3) and 626/55 (DENV4) showed excellent specificity. The 323 mAb recognized DENV serotypes 1–4; however, we found that when used as a 323/323 homo-pair, the detection of dengue serotype 4 NS1 in the pan-dengue test was not optimal. Therefore, by conjugating a mixture of mAb on the pan antibody nanoparticles (271, 243, 411, 626), we achieved improved limit of detection results in the pan-DENV dipstick test. The linear peptide mapping data provide an important framework for understanding the mechanisms of serotype-specific DENV detection and differential detection of DENV and ZIKV.

Laboratory validation of the rapid tests

The rapid diagnostic test reported here is an immunochromatography format with visual readout using anti-NS1 antibodies that are either coupled to gold nanoparticles or adsorbed to nitrocellulose membranes. Each test strip has a positive control area adjacent to the paper absorbent pad or wick; the positive control is an anti-mouse Fc domain antibody that will capture any of the antibody-conjugated gold nanoparticles to generate a positive control visual signal (Fig. 2). Red/purple spots or stripes at the NS1 test area reflect NS1 protein that is "sandwiched" between two antibodies; that is, a capture anti-NS1 antibody adsorbed to the nitrocellulose membrane at the control or test area, and a second anti-NS1 antibody covalently coupled to visible gold nanoparticles. To test for binding specificity and cross reactivity with each of the antibody pairs, dipsticks 1–4 (detecting DENV serotypes 1–4), pan-dengue (P, detecting all four DENV serotype NS1 proteins), or ZIKV (Z) were chromatographed using solutions of the following recombinant NS1 proteins (500 ng/ml): DENV serotypes 1–4, ZIKV, West Nile virus (WNV), Yellow Fever Virus (YFV), Tick Borne Encephalitis virus (TBEV), and Japanese Encephalitis virus (JEV). Figure 2A shows DENV serotypes 1–4, and Fig. 2B shows pan dengue and ZIKV strips. The results demonstrate that specific NS1 signals were observed for each of the DENV serotype NS1 proteins on the appropriate strip (Fig. 2, arrows). Strip 1 detected DENV 1 recombinant NS1 protein expressed by mammalian cells (rNS1), with no detectable interaction with the other DENV serotype proteins or with other flavivirus rNS1 proteins. Similar results were observed with strip 2, where DENV2 NS1 was detected; also with strip 3, where DENV3 NS1 was detected, and with strip 4, where DENV4 NS1 was detected.

Figure 2B, dipsticks "P" show, the performance of the pan-dengue strip that detects all four DENV serotype NS1 proteins. The "Z" strips demonstrate the performance of the ZIKV strips. To prepare the pan-dengue nanoparticles, four mAbs (271, 243, 411, and 626) were covalently coupled as a mixture to gold nanoparticles, and mAb 323 (Fig. 2B and table S4) was adsorbed to the nitrocellulose membrane. The data demonstrate that all four DENV serotype proteins were detected by using the pan-DENV strip. Fig. 2B (right) also shows the ZIKV strip detecting recombinant ZIKV NS1 protein (strips "Z"). These results indicate that the DENV and ZIKV strip are specific in detecting recombinant DENV serotype NS1 proteins or recombinant ZIKV NS1 without cross reactivity interference from other flavivirus NS1 proteins.

The limits of detection for the pan-dengue, serotype-specific DENV, and ZIKV NS1 tests were defined by chromatographing dilutions of recombinant NS1 proteins and then quantifying the signal intensities, normalizing them to the plateau maximum, and plotting sigmoidal data fits (Fig. 2C–2E). The limits of detection were calculated as the NS1 concentrations intersecting a line representing a signal intensity point 5-fold greater than the standard deviation of the background signal. The results show that the limits of detection for the DENV serotypes 1–4 NS1 proteins ranged from ~4 ng/ml to 21 ng/ml, whereas the range was 1–11 ng/ml for the pan-dengue strip. These limits of DENV NS1 detection are far below the reported concentrations of DENV NS1 protein in serum (up to 50 μg/ml) (2). The limit of detection for the ZIKV strip was 18 ng/ml. Serum NS1 concentrations in ZIKV-infected patients have not been reported. For comparison with current NS1 rapid tests, we

chromatographed DENV NS1 proteins on dengue NS1 rapid tests from Standard Diagnostic (fig. S3). The results suggest that limits of detection for the dengue rapid tests that we developed (described in Fig. 2) are under 75 ng/ml, whereas the limit of detection for the Standard Diagnostic tests is between 75 ng/ml and 150 ng/ml.

As a final validation step preceding patient sample analysis, the dipstick strips were tested in the laboratory by chromatographing native NS1 protein released by virus-infected Vero cells into cell culture supernatants (Fig. 2F). Although the recombinant NS1 proteins used in Fig. 2 were expressed by eukaryotic cells to optimize antigen protein folding with secondary modifications such as glycosylations, testing the binding of native NS1 proteins released by virus-infected cells is a more robust proxy for analyzing clinical serum samples. Vero cells were infected individually with DENV serotypes 1–4, or with ZIKV (MR766 Uganda or contemporary Asian/American strains). Cell culture supernatants were collected and chromatographed on the dipsticks in a manner similar to Fig. 2A. The data in Fig. 2F show the recognition of test dipsticks 1–4, as well as the pan-dengue strip, with each of the DENV 1–4 Vero cell supernatants. These results are evidence that native NS1 protein expressed by DENV-or ZIKV-infected Vero cells yielded positive signals on the corresponding DENV 1–4, pan-dengue, and ZIKV dipstick tests, without detectable cross reactive binding (arrows).

As a comparison with current commercial DENV rapid tests, we chromatographed NS1-containing supernatants from ZIKV-infected Vero cells on Standard Diagnostic dengue rapid tests. In contrast to the lack of cross reactivity observed in Fig. 2F, the Standard Diagnostic test showed cross reactivity with the Vero cell supernatant (fig. S4, lanes 1–4). To extend these tests, we also chromatographed recombinant ZIKV NS1 protein on a Standard Diagnostic test. The results (fig. S4, lane 7) are consistent with the Vero supernatant results (lanes 1–4), confirming that the Standard Diagnostic test shows cross reactive binding when ZIKV NS1 protein from ZIKV-infected Vero cells or recombinant ZIKV NS1 (500 ng/ml) was run. These results support a premise of this work: that consideration of DENV/ZIKV NS1 cross reactivity is essential in evaluating rapid diagnostics.

Validating the immunochromatography tests using clinical serum samples

We next turned to detecting ZIKV and DENV NS1 in a retrospective study of de-identified surveillance serum samples from human patients. Serum samples were tested in Brazil, Mexico, Colombia, Panama, Guatemala, and India (Fig. 3A) following approved human subjects use protocols. The sera were banked frozen samples representing blood that was drawn from febrile patients during routine care. Prior to using the rapid tests, the DENV and ZIKV serum samples were validated by RT-PCR, and positive tests were also evaluated using a lab-generated ELISA. The DENV-pan ELISA (Fig. 3B, left panel) was validated by testing supernatants of Vero cells infected with molecularly defined DENV or ZIKV isolates. As expected, the supernatants from DENV infected Vero cells gave high signals in the DENV ELISA (lane 1) but no signal in the ZIKV ELISA (lane 6); ZIKV NS1 was detected from ZIKV infected cell supernatant in the ZIKV ELISA (lane 7), but did not cross react in the DENV ELISA (lane 2). Serum samples from uninfected patients were not recognized in the ELISAs (Fig. 3B, lanes 3 and 8). However, serum samples from DENV-infected patients yielded strong ELISA signals in the DENV ELISA (lane 4), but not in the

ZIKV ELISA (lane 9). Conversely, sera from ZIKV-infected patients did not yield signal in the DENV ELISA (lane 5), but gave clear signals in the ZIKV ELISA (lane 10). These results strongly suggest that the validated ELISA detected DENV and ZIKV NS1 proteins without cross reactivity interference. In the analysis of twenty-five ZIKV infections and fifty-one DENV infections, the serum NS1 concentrations were found to be lower in ZIKV-infected patients (lane 10) than in DENV-infected patients (lane 4). The concentrations of NS1, approximately 30 ng/ml ZIKV and 120 ng/ml DENV, are consistent with other reports of low ZIKV viremia (16). The availability of the DENV and ZIKV ELISAs (Fig. 3B) allowed us to validate ZIKV serum samples for subsequent testing in the dipstick rapid tests.

Representative serotype-specific DENV detection using de-identified surveillance serum samples is presented in Fig. 3C. Each sample was tested using a panel consisting of five strips, with strips 1–4 recognizing DENV serotypes 1–4, respectively, and strip P recognizing all four serotype NS1 proteins (pan-dengue, confirming DENV infection). Although the specific serotype signals are visually apparent, to remove any subjectivity in reading and interpreting the results we captured images of the test strips using a mobile phone camera and performed automated image recognition and processing using ImageJ to objectively quantify the data. Fig. 3D–3G demonstrate that that each of the specific serotype signal signals was statistically distinct from the other serotype signals ($P < 0.001$). A representative example of serum from a ZIKV-infected patient chromatographed on the ZIKV dipstick (Fig. 3C, right; ZIKA) shows the serum sample (Z) is positive but serum from an uninfected patient (θ) is negative. Quantification in Fig. 3H also demonstrates test signal intensities were distinct from uninfected patients.

To test for DENV/ZIKV NS1 cross reactivity, supernatants from Vero cells infected with DENV serotype 4 were chromatographed on DENV serotype specific dipsticks 1–4, the pan dengue dipstick (P), as well as the ZIKV NS1 dipstick (Z). Supernatant from Vero cells infected with DENV serotype 4 was detected, as expected, in strip 4 and the pan strip (P) (arrows); however, there was no detectable signal in the ZIKV strip (Fig. 3I). Comparable experiments were run with supernatants from cells infected with the other three DENV serotypes and the expected results were observed (Fig. 3I). Alternatively, when supernatant from C6/36 insect cells that were infected with ZIKV (strain NR-50210, Human 2016 Panama) was tested, there was no signal in strips 1–4 and P, but positive signal in strip Z (Fig. 3J, arrow). ZIKV nucleic acid has been detected in semen (17), and DENV NS1 has been detected in urine (18, 19); however, the presence of ZIKV NS1 in the urine of infected patients has not been confirmed. We obtained three paired patient samples of acute PCR-positive ZIKV serum and urine and concentrated each five-fold using a centrifugal filter. The chromatography results show positive signals and detection of ZIKV NS1 in the serum samples (Fig. 3K, strips labeled "S" at top), but no signal in any of the urine samples (strips labeled "U" at top). Quantifications in Fig. 3L and 3M, indicate that the ZIKV and DENV NS1 antigen strips do not cross react. Quantification of NS1 from five serum/urine pairs is shown in Fig. 4N. Overall, the results presented in Fig. 4 demonstrate serotype-specific DENV NS1 and ZIKV NS1 detection in clinical serum samples, without detectable cross reactivity interference.

Rapid test detection window

Rapid test NS1 detection was correlated with days post-onset of symptoms when disease metadata were available. Because the DENV human serum samples used in this study were retrospective frozen surveillance samples, metadata were not available in all cases. Metadata from DENV samples from India and Colombia, as well as ZIKV samples from Guatemala and the Dominican Republic included the fever onset day and fever duration. Normalized rapid test signals for DENV and ZIKV detection are presented in fig. S5A and fig. S5B, respectively. The results suggest that the DENV rapid tests detected DENV NS1 at days 2–5 post-onset of symptoms, in agreement with published results (6). The ZIKV test detected ZIKV NS1 at days 2 to 8 (fig. S5B); however, greater numbers of samples and controlled clinical studies will be needed in future work to confirm the detection window.

Test data were analyzed further to define sensitivity (identifying true positives; individuals who have disease) and specificity (true negatives; individuals without disease). We evaluated sensitivity and specificity as a function of an intensity cutoff value, above which a test was scored positive and below which a test was considered negative. Receiver Operator Characteristic (ROC) curves illustrate the performance of the rapid tests as a function of the discrimination threshold, plotted as sensitivity vs 1-specificity in Fig. 5A; numerical values are shown in Fig. 5B. The areas under the ROC curves are a proxy of test performance, where 1 represents a perfect test and 0.5 a random predictor. We measured areas of 0.88, 0.96, 1, 0.98, 0.95 and 0.82 for DENV1, DENV2, DENV3, DENV4, Pan DENV, and ZIKV, respectively. The calculated optimal cutoff values were 1.14, 1.18, 1.12, 1.37, 1.9 and 1.08 for DENV1, DENV2, DENV3, DENV4, pan-DENV, and ZIKV, respectively, using a bootstrap technique with 1000 iterations. Sensitivity is defined as the number of measured positives divided by the total confirmed positives, and specificity is defined as the measured negatives divided by the total of confirmed negatives. Using the optimal cutoff value, the test sensitivity and specificity were: 0.76 (13/17) and 0.89 (33/37) for the DENV1 test; 0.89 (8/9) and 0.98 (44/45) for the DENV2 test; 1 (16/16) and 1 (39/39) for the DENV3 test; 1 (6/6) and 0.96 (44/46) for the DENV4 test; 0.88 (51/58) and 1 (11/11) for the pan-DENV test; finally, 0.81 (25/31) and 0.86 (6/7) for the ZIKV test.

Discussion

Rapid diagnostics provide critical information that informs patient care and assesses patient risks, generally within one hour after sample collection. Despite urgent needs, efficacious and inexpensive rapid tests are not available for many infectious diseases, including arboviruses (mosquito and tick-transmitted viruses). Viral antigen-based tests provide important patient benefits in geographic areas where molecular tests such as RT-PCR are not available, have very slow turnaround times, or are prohibitively expensive. Rapid tests have further applications in the developed world, where immediate results may be required in the context of febrile travelers at airports or for military personnel serving in endemic areas. Accurate pathogen identification is essential, a goal that is complicated when distinguishing closely related co-circulating viruses such as DENV and ZIKV, which have markedly different risk profiles. The similarity of clinical symptoms is a compounding problem in many cases, and also a justification for improved diagnostics that provide accurate data on

which to base clinical decisions. DENV, ZIKV, and Chikungunya (CHIKV, an alphavirus) infections exhibit common symptoms including varying degrees of fever, headache, myalgia, arthralgia, nausea, and rash, with hemorrhagic fevers as outcomes of severe infections in about 1% of infected individuals (24). However, the ZIKV outbreak stunned the virology and medical communities with a distinct set of risks and severe outcomes not previously associated with flavivirus infections, including fetal microcephaly and Guillain-Barre syndrome. Because DENV and ZIKV have major impact on economically challenged countries in the tropics, our approach has been guided by the World Health Organization acronym "ASSURED" to describe the ideal characteristics of a diagnostic test that can be used at all levels of the healthcare system: affordable, sensitive, specific, user-friendly, rapid, equipment-free, and delivered to those who need them (25, 26).

This report describes DENV rapid tests that specifically detect the viral NS1 protein to identify and distinguish the four DENV serotypes without observed cross-reaction with ZIKV. Despite the high percentages of homology and identity among the flavivirus NS1 proteins (table S2), our screening strategy (fig. S1; table S1) identified antibody pairs that detected and distinguished the four DENV serotype NS1 proteins, as well as ZIKV NS1 (recombinant and expressed by virus-infected cells), without detectable cross reactive interactions. These results are important for several reasons. First, we have identified antibody pairs that distinguish closely related NS1 proteins, and applied them to create rapid tests. Success in detecting/distinguishing the individual serotypes was dependent on a) immunizing groups of mice with the native dimer/hexamer forms of recombinant NS1 that were expressed by eukaryotic cells; b) initiating hybridoma screening against multiple NS1 proteins in parallel, enabling us to use ELISA and flow cytometry methods to sort and select from the most diverse pool; and c) performing systematic unbiased pairwise screening of the selected clones to identify specific antibody pairs to recognize specific serotypes (table S3). Similar approaches may be used to identify and distinguish other closely related proteins. Second, epidemiological surveillance is a second area of importance for serotype-specific detection, and is a critical component of patient care and public health preparedness (16, 27, 28). The serotype specific tests described here could be used in inexpensive patient screening during routine medical care to identify not only incoming serotypes, but also patients in endemic areas who may have asymptomatic infections. The introduction of an additional DENV serotype into a region may cause outbreaks with severe hemorrhagic fever presentation because the individual serotype infections do not provide long-lasting cross-protection and heterologous infections increase the hemorrhagic fever outcome of dengue. Therefore, we propose that rapid testing may diminish the impact of emerging epidemics by enabling early detection of outbreaks. Third, serotype-specific detection has implications for DENV vaccine development because current vaccine candidates may not show equal protection across the serotypes (29), and knowledge of circulating serotypes can inform trials and outcomes. Finally, the approaches for rapid tests described here represent a platform that can be applied toward detecting the future emergence of new pathogens. Kuno *et al.* (30)_report approximately seventy viruses in the genus *Flaviviridae* alone, and the methods described here can be applied toward detecting and distinguishing other potentially emergent flaviviruses while minimizing cross reactivity.

In addition to detecting and distinguishing DENV serotypes, we report here a rapid test for ZIKV NS1. Laboratory testing demonstrated that the ZIKV NS1 test does not cross react with DENV NS1 (Figs. 2 and 3; table S4). Detection without cross-reaction was also confirmed using patient samples (Fig. 4). Detecting ZIKV NS1 in patient samples was more challenging than detecting DENV NS1 because ZIKV NS1 concentrations were about 10-fold lower than DENV patient NS1 (Fig. !!!3). We addressed this by concentrating the sera using a centrifugal filter (a 5-minute centrifugation at 10,000g). As shown in Fig. 4, ZIKV NS1 was detectable in samples that were concentrated 5-fold; that is, to 30 microliters from a starting volume of 150 microliters. Although urinary DENV NS1 has been reported (3, 31), ZIKV NS1 was not detected here in 5X concentrated urine samples, either by the ELISA or rapid tests. We cannot rule out the possibility that urinary ZIKV NS1 is present at concentrations that are below detection by our assays.

The use of image processing and computer software to analyze rapid tests has been described previously in non-clinical analysis (32, 33). Mobile image capture and image processing ensures objective and quantified data under varied use conditions and signal intensities, and generates standardized data that can be shared and compared on a global basis. Tests showed both very high intensity signals, especially with DENV detection, and also low intensity signals, often with Zika virus NS1 (Figs. 3 and 4). However, low signal intensity is not restricted to ZIKV because DENV NS1 signals can also be very low immediately after the onset of disease symptoms (days 0–1) and also approximately 6–7 days after onset of symptoms, when the virus is being cleared (34). Although the human eye is extremely sensitive, machine vision offers improved performance for quantitative measurement (35). Andries *et al.* reported wide differences in sensitivity and specificity when different people at different sites evaluated the same diagnostic. These observations suggest that training and oversight are required in the use of rapid tests (36). Image processing and data quantification as described here, avoid errors in user-based interpretation. The approach is independent of computation capacity of the phone because the computation is performed "in the cloud". Immediate internet connectivity is not required in field applications of the tests because our store-and-forward software enables the coded de-identified image data to be stored on the phone and uploaded or transferred at later times for analysis. The ubiquity of mobile phones allows test users to obtain an objective analysis based on a common algorithm shared across a global network.

The DENV and ZIKV tests described here compare very well with rapid tests described previously (4, 6, 7, 9, 36–38), with the added crucial benefit of avoiding detectable cross reactivity between ZIKV and DENV and among ZIKV/DENV and a number of other flavivirus NS1 proteins. Nonetheless, the current DENV and ZIKV tests described here have some limitations, and opportunities for improvement remain. Using whole blood rather than serum would simplify use as a point of care diagnostic in the clinic. The tests reported here were performed using serum because the clinical samples available for retrospective analysis in the endemic areas were frozen serum samples that were validated for virus infection by RNA extraction and nucleic acid amplification and/or by ELISA for the NS1 protein. For whole blood analysis, we have developed and successfully tested lateral flow chromatography devices that incorporate our antibodies and use a specialized sample pad paper that removes red blood cells; therefore, we do not anticipate the use of blood as an

obstacle for future versions of the rapid tests. The sensitivity and specificity of the ZIKV tests ranged from 0.7–1.0. The sample availability for Zika testing was very limited, due to the fact that ZIKV is an epidemic that only recently emerged. Further ZIKV testing and device optimization will be possible as the availability of patient samples improves. The current state of research is that it is very difficult to obtain validated acute phase ZIKV patient samples with complete metadata, a challenge that is also reflected in single sample data points reported recently from the Florida, USA ZIKV outbreak (20). Viral NS1 concentrations vary in individual patients and with time post-infection (21). ZIKV test performance was improved by concentrating serum samples. Although centrifugation often requires instrumentation and power, in contradiction with the "ASSURED" paradigm, unpowered "whirlygig" centrifugation devices have been described recently (39), which could concentrate samples or separate serum while meeting the "ASSURED" criterion of "equipment free". The concentration step was not needed to detect serum ZIKV NS1 using the lab-made ELISA with 150 microliters of serum. A rapid test that could accommodate a 150 microliter sample volume (currently limited by the wick absorption) may permit greater sensitivity, although it would likely be necessary to increase the number of conjugated nanoparticles used in the test.

Device cost and detection during secondary infections are important issues to consider. The current cost for each strip (Figs. 3 and 4) is nearly $5.00, which is due primarily to using commercial gold nanoparticles, coupled with small-scale antibody production. The cost of the nanoparticles can be decreased by 1000-fold by using lab-made nanoparticles that have excellent performance (fig. S6); antibody production scale-up will further decrease costs. We considered the possibility that the efficacy of our rapid tests for detecting NS1 might be compromised in secondary dengue infections, where circulating anti-NS1 antibodies could bind NS1 to form immunocomplexes that would shield NS1 from recognition by the rapid test antibodies. Using our DENV diagnostic, we tested a number of confirmed secondary dengue infection samples that had been confirmed by serological rapid tests and hemagglutination tests that established relative IgG/IgM concentrations. We successfully detected DENV NS1 protein in secondary infections (fig. S7), consistent with a recent report (40). We speculate that detecting NS1 in secondary dengue infections might be more feasible than detecting envelope (E) protein because the interfering anti-E antibodies are present in larger quantities in the serum and in higher amounts compared with the anti-NS1 antibodies. Therefore a test based on E detection may be blocked via competition between serum polyclonal antibodies and the monoclonal antibodies used in the nanoparticle.

The rapid NS1 antigen test described here is effective only when analyzing samples collected during the acute phase of the virus infection, when flavivirus RNA and NS1 are detectable, prior to virus clearing by the immune system. Serological tests for anti-envelope protein IgG/IgM or anti-NS1 protein IgG/IgM are useful for evaluating patients post-acute phase. Several companies are marketing DENV and ZIKV IgG/IgM tests, although in many cases their cross-reactivity has not been evaluated. A new approach, reported recently, shows high sensitivity and specificity for ZIKV immunoglobulins (41). We propose that optimal patient care will be provided through use of several diagnostic approaches that are applicable for a range of clinical needs. Rapid immunochromatography tests are well suited for fast turnaround times without need for specialized reagents, equipment, or trained personnel.

Rapid tests are generally low-cost, can often be transported without refrigeration, and can be used in austere environments. Nucleic acid amplification methods are highly specific with low limits of detection as compared to immunochromatography strips; however, disadvantages include requirements for equipment powered by electricity/batteries, specialized reagents, and a cold chain for maintaining enzyme activity. Synthetic biology approaches (42) may offer a hybrid approach wherein the simplicity of paper diagnostics can be combined with isothermal nucleic acid amplification. There is great need for all of these technologies in order to improve the time-to-diagnosis in patients infected by pathogens worldwide.

In summary, here we identified monoclonal antibody pairs and developed rapid tests that detect and distinguish DENV NS1 antigen for serotypes 1–4, pan-dengue, and ZIKV NS1 without detectable cross reactivity. We report the limits of detection of each antibody pair, demonstrating that DENV NS1 can be detected using serotype-specific tests and pan-dengue tests in the 1 ng-20 ng/ml range and ZIKV NS1 detection at about 20 ng/ml. The strips have been validated using recombinant NS1 protein, NS1 present in the supernatants of virus-infected Vero cells or C6/36 cells, and NS1 protein present in clinical serum samples from several geographic areas in the Americas and India. The use of a mobile phone camera linked to ImageJ analysis of the rapid tests permitted objective analysis of the test data. Taken together, we demonstrate that we have developed and characterized NS1 antigen tests capable of distinguishing the DENV serotypes and detecting ZIKV and DENV infections without cross reactivity, in anticipation of broader clinical applications.

Materials and Methods

Study Design

The overall objective of the study was to develop and validate a platform approach for producing low cost paperfluidic rapid antigen tests to detect viruses while minimizing cross reactivity and false positive signals. The platform approach centers on screening monoclonal antibodies against panels of multiple flavivirus NS1 antigens in order to assess binding specificity, relative binding affinity, and cross reactivity. Dengue and Zika viruses are the focus of this report; however, the approach can be used with any of the estimated 70 flavivirus NS1 proteins. Monoclonal antibody linear epitope binding sites were defined, and the antibodies were tested pair-wise in dipstick tests to identify those that yielded specific NS1 binding without detectable cross reactivity. Limits of detection were defined using recombinant NS1 proteins. The dipstick tests were validated in endemic areas using de-identified serum samples that were collected from febrile patients. The majority of testing was performed on-site in Central America, South America, and India. The serum samples were validated using nucleic acid amplification methods and NS1 ELISAs to generate validated test panels. To remove subjectivity from the test result readings, mobile phone cameras were used to capture test strip images, and the test signals were quantified using ImageJ. These quantitative data were then used to generate test sensitivity and specificity values using receiver operator curve (ROC) analysis.

Monoclonal antibody production strategy

DENV and ZIKV anti-NS1 monoclonal antibodies were produced in mice under contract (Covance, Inc), following an approved animal care protocol. BALB-c mice were immunized with purified recombinant DENV or ZIKV NS1 proteins that were expressed in mammalian cells (Native Antigen Co.) to facilitate protein folding and post-translational modifications. From each of the immunized groups, one seroconverted animal with high titers of antibodies recognizing a pool of the NS1 DENV antigens was used for cell fusion to generate hybridomas. Supernatants from cloned hybridomas were tested by ELISA to generate "fold over background" values that gave us an approximate idea of serotype specificity or DENV/ZIKV specificity (see table S4). From the ~200 DENV hybridomas and 100 ZIKV hybridomas identified in the initial screening (table S1), supernatants from 30 DENV clones and 16 ZIKV clones were used to stain virus-infected Vero cells, followed by analysis in flow cytometry. This step was included to ensure that the selected hybridomas produced antibodies that recognized native NS1 protein expressed by virus-infected cells. Approximately 15 DENV anti-NS1 hybridomas and 16 anti-ZIKV NS1 hybridomas were expanded by grown in low IgG serum (Invitrogen) containing hybridoma cloning supplement (Roche). The expressed monoclonal antibodies were isotyped and purified by affinity chromatography on a protein L matrix (GE Healthcare). Purified antibodies were concentrated and buffer exchanged into PBS. Eleven purified DENV antibodies were then tested pairwise in immunochromatography tests, as were 10 ZIKV antibodies to identify pairs that exhibited high differential NS1 binding and low non-specific background interactions.

Anti-ZIKV monoclonal antibodies were generated using a modified immunization and B cell harvest protocol. Both a rapid lymph node approach (B cell isolation from lymph nodes and cell fusion at 23 days after immunization) and the more traditional spleen B cell hybridoma method (B cell isolation from B cells and cell fusion at 105 days post-immunization) were used. To select antibodies with minimal potential for cross reactive DENV NS1 recognition, an enhanced screening approach was also applied. Approximately 100 anti-ZIKV NS1 hybridoma supernatants were screened by indirect ELISA against not only ZIKV NS1 protein, but also DENV serotypes 1–4 NS1 proteins, as well as a panel of flavivirus NS1 proteins including West Nile Virus, Usutu Virus, Tick Borne Encephalitis Virus, and Japanese Encephalitis virus. The antibody distribution data (fig. S2, right) demonstrate that the proportion of ZIKV NS1-specific antibodies was higher for the lymph node fusion approach than the traditional spleen cell fusion method. From the starting pool of about 100 anti-ZIKV mAbs, 16 anti-ZIKV were selected for step 2, flow cytometry analysis.

Antibody conjugation to nanoparticles

40 nm gold nanoparticles were purchased from Innova Bioscience. The serotype-specific DENV nanoparticle-antibody conjugates and the ZIKV nanoparticle antibody-conjugates were prepared according to manufacturer's instructions. For single antibody conjugations, the antibody was first diluted to 0.1 mg/ml in the supplied dilution buffer. Next, 12 μl of diluted antibody was mixed with 42 μl reaction buffer. 45 μl of the mix was then used to suspend the lyophilized gold nanoparticles (20 OD particles). The antibody-nanoparticle mix

was incubated for 10 minutes at room temperature, followed by the addition of 5 μl of proprietary quencher solution to stop the coupling reaction. After adding the quencher solution, 100 μl of 1% Tween-20 in PBS and 50 μl of 50% sucrose in water were added to the conjugates prior to use in immunochromatography. The "pan dengue" nanoparticles used for detecting all four DENV serotypes were conjugated using a mixture of four different antibodies. These particles were created by preparing 12 μl of solution containing each of the four antibodies (MA724.271, MA7732.243.108, MA724.411, and MA726.626) at a final concentration of 0.1 mg/ml (final total antibody concentration 0.4 mg/ml). 12 μl of the resulting antibody mixture was then mixed with 42 μl of reaction buffer and processed according to the same protocol as the other conjugates.

Antibody application to nitrocellulose membranes

Nitrocellulose membrane (EDM Millipore HF18002XSS) was cut into strips using a laser cutter (Universal Laser Systems; model VLS2.30; 30 watt) at 30% power and 90% speed. The strip pattern was designed in Adobe Illustrator. Strips were attached to a wick (GB003 Gel Blot Paper) with adhesive paper (MIBA-010 Backing Card, 0.020" thickness; DCN Diagnostics. For the positive control area, 0.33 μl of anti-mouse Fc antibody (EDM Millipore AQ127) at 1 mg/ml was spotted on the control line. The anti-NS1 capture line on the nitrocellulose was generated by pipetting 0.33 μl of anti-DENV NS1 antibody (4 mg/ml) at the NS1 test area. Strips were air-dried and stored in a dessicator at room temperature before use.

Immunochromatography

Each immunochromatography strip was run in a separate microcentrifuge tube, and groups of tubes/strips were run together. The rapid test solution contained 1) 30 μl of human acute DENV/ZIKV/uninfected serum sample, 2) 30 μl of fetal calf serum, 3) 5 μl of proprietary quencher solution (Innova Inc.), and 4) 16 μl of conjugated gold nanoparticle mix (see Antibody conjugation to nanoparticles). The run time varied with the sample and humidity; it was always less than one hour and sometimes as short as 15 minutes. The strips were left to dry and then imaged for quantitative signal analysis.

ELISA for the detection and quantification of circulating ZIKV and DENV NS1

96-well plates (Costar, Flat bottom high binding; Cat: 3590) were coated with 100 μl/well of optimized doses of Mab 110 diluted in 1X PBS (Gibco, Ref: 10010–023) and incubated overnight at room temperature (RT). After blocking with 5% non-fat dry milk (Chem Cruz, Cat: sc-2325) 0.05% Tween 20 (Sigma-Aldrich, Cat: p-1379) in 1X PBS (5% blotto) for 1 hour, serial dilutions of rNS1 from ZIKV, DENV, Yellow Fever Virus (YFV), West Nile Virus (WNV), Japanese Encephalitis Virus (JEV), Tick-Borne Encephalitis Virus (TBEV) (all starting at 2000 ng/mL in 2.5% blotto), or undiluted serum/plasma samples or supernatant of ZIKV or DENV-infected Vero cells were added and incubated for 2 hours at RT. To detect ZIKV NS1, the plates were washed three times with 0.1% Tween 20 in 1X PBS (wash buffer) followed by the addition of 100 μl/well of optimized doses of biotin-labeled mAb 130 diluted in 2.5% blotto, followed by incubation for 1 hour at RT. After another washing step, 100 μl/well of 1 μg/ml of peroxidase-labeled streptavidin (Thermo Scientific, Cat: 21130) was added and the plates incubated for 1 hour at RT. Finally, the

plates were developed with tetramethylbenzidine single solution (Life Technologies, Cat: 002023), and the reaction stopped with 50 μl/well of 2M sulfuric acid (RICCA Chemical Company, Cat: 8315–32). A Tristar LB 941 spectrophotometer (Berthold Technologies) was used to read the plates at a wavelength of 450 nm. In the quantitative ELISA for the detection of DENV NS1, a protocol similar to ZIKV NS1 ELISA was followed, using the anti-DENV NS1 mAb 323 as capture antibody and the biotin-labeled MAb 323 as detection antibody. A standard curve was generated with addition of mixed recombinant NS1 from DENV 1–4 (Native Antigen Co.), starting at 2000 ng/mL. The samples were diluted 1/100 in 2.5% blotto and run in duplicate. The mean optical densities (OD) 450 nm of the negative controls (samples with previously known undetectable NS1) were 0.12 and 0.15 for ZIKV and DENV NS1 ELISA, respectively. Wells where only 2.5% blotto was added had similar OD 450 nm. The limit of detection of the assays was taken as the concentration of ZIKV and DENV rNS1 with at least two-fold higher OD 450 nm than the background (samples with undetectable NS1). When analyzing clinical samples, the correlation coefficients of the standard curves of recombinant ZIKV and DENV NS1 were ≥94% and the duplicate variability was <10%. Calculating the concentration of ZIKV NS1 was done by interpolation of the OD 450 nm of the samples to the standard curve using 4-parameter logistic regression with GraphPad Prism software version 7.0.

Patient Serum Samples

Each of the collaborating laboratories contributed a panel sample size of 6–20 de-identified retrospective serum samples. MIT IRB and local IRBs approved the protocols for purposes of pathogen identification. Virus identity in human serum samples was confirmed by nucleic acid amplification (RT-PCR). Dengue infections were confirmed using Lanciotti primers (43). Primer and probe sequences for ZIKV were provided by CDC, Puerto Rico; 1086f ZIKV 5'CCG CTG CCC AAC ACA AG 3'; 1162 ZIKV 5' CCA CTA ACG TTC TTT TGC AGA CAT 3'; 1107-FAM 5' FAM AGC CTA CCT TGA CAA GCA GTC AGA CAC TCA A. The serum panels used for the retrospective rapid test analysis of DENV and ZIKV acute sera were stored at each of the collaborating laboratories at −80°C until use. To use the serum samples in immunochromatography, the serum was first passed through a Spin-X Centrifuge tube filter with 0.2 um Nylon filter (Costar, catalog #8169) to remove any cryo-aggregates. For ZIKV detection, 150 ul of cleared filtered serum was then 5X concentrated using a centrifugal filter (Amicon ultra 0.5 ml 30K, Catalog # UFC503096) that was centrifuged for 10 minutes at 13000 rpm using in an Eppendorf tube centrifuge. If sufficient volume of the serum sample was available (100 μl,) it was analyzed using the laboratory ELISA as described above. A sample size of 30 ul of DENV serum, or 30 μl of 5X concentrated (from 150 microliters) ZIKV sample serum or urine, was used for the rapid tests.

Image Analysis

Rapid test results were analyzed using image processing software to machine-read and quantify test results. After completion of the test run, the strips were taped to a sheet of paper with a printed red box outline that was slightly larger than the strip length. Within the red box, short black vertical lines served as fiduciary markers for image processing, separating the strips and identifying top/bottom. The image of the strip inside the red box

was captured with a mobile phone camera and analyzed using an ImageJ macro to quantify test results. ImageJ is public domain image analysis software. The ImageJ macro provided instructions to localize and scan the positive control area and test area in each test. Briefly, a Workflow (DeskConnect, Inc.) script was written to run on the mobile phone, with instructions to GPS-localize where the image was captured, followed by uploading to Dropbox. A Python script running on a desk computer was activated upon Dropbox sync, calling ImageJ to perform the image analysis on the uploaded file. An ImageJ macro identified the red square and black line fiduciary markers, followed by drawing a rectangle in between the black markers, on the strips. ImageJ quantified the signal at the test area and blank/background area, and generated a normalized intensity, calculated by dividing the maximum intensity value at the test band by the average value of a blank area in each test strip. Test data were stored and used later for sensitivity/specificity and ROC curve analysis.

Limits of detection

Limits of detection for each of the antibody pairs were measured using recombinant NS1 proteins antigens. Antigen solutions were diluted serially and chromatographed on DENV 1–4 dipsticks, the pan-dengue dipstick (P), or the ZIKV dipstick (Z). The signal intensities were quantified (ImageJ), normalized by the intensity at the highest concentration, plotted against antigen concentration, and fit to a sigmoidal curve. The limit of detection was calculated from the sigmoidal curve fit as the NS1 concentration found at the intersection with a line representing the value of 5-times the standard deviation of background signal intensity. Background was determined by analyzing triplicate strips in nitrocellulose areas outside of the test and positive control regions.

NS1 detection in secondary dengue infections

To define primary and secondary dengue infection samples, paired acute/convalescent serum samples from a Venezuelan cohort (44,45) were tested using a standard hemagglutination assay (4,46) where virus particles bind to sialic acid residues on the surface of red blood cells, causing the red cells to agglutinate. Serum dilutions are added to the assay, and anti-virus antibodies present in serum bind to virus particles and thereby inhibit the agglutination. The dilution factor marking the interface of hemagglutination inhibition/no inhibition is a measure of anti-virus (specifically anti-envelope protein) antibodies. The World Health Organization has defined primary dengue infections as those with hemagglutination inhibition antibody titers less than 1:640, while titers of 1:1280 or higher signal secondary dengue infections. For the work reported here, diluted patient serum samples from dengue-infected patients were mixed with red blood cells, and agglutination status was scored. Samples with antibody titers >1280 were defined as secondary infections and analyzed for NS1 detection using the rapid tests.

Receiver Operator Curve (ROC) Analysis (ROC curves)

The individual rapid tests (DENV 1–4; pan-DENV; ZIKV) were validated using PCR- and/or ELISA-confirmed serum samples containing variable NS1 protein concentrations, resulting in normalized test signal intensities (test signal intensity/background signal intensity) ranging from 0.5 to 5. Based on these data, MATLAB was used to plot and calculate ROC curves, as well as to find the optimal cutoff values for each rapid test type

(DENV 1–4; pan-DENV; ZIKV). In practical use, rapid tests with normalized intensity values greater than the cutoff are defined as positive, while those with normalized intensity values less than the cutoff are negative. To generate the optimal cutoff values and ROC curves, ImageJ was used to scan each of the test strips, yielding a signal intensity for each test area and also for background in an area of the strip outside of the test and control areas. These normalized intensity values were inputs to a MATLAB script. The script used theoretical cutoffs between 0.5 to 5, and calculated both the sensitivity (number of true positives divided by total confirmed positive values) and specificity (number of true negatives divided by the total confirmed negatives) at each of the theoretical cutoffs. After calculating the sensitivity and specificity at each theoretical cutoff, the optimal cutoff value was defined at the highest sum of sensitivity and specificity. In this way, the optimal cutoff value reflected the optimal sensitivity and specificity performance of the test. To calculate the confidence intervals, the ImageJ intensity measurements were fitted with a generalized linear regression model with binomial distribution and a link logit function, using MATLAB's fitglm function. MATLAB's perfcurve function with a bootstrap of 1000 was used to evaluate the linear regression model and calculate the 95% confidence intervals of the areas under the curve, sensitivity and specificity. The optimum cutoffs obtained for patient samples were 1.14, 1.18, 1.2, 1.37, 1.19 and 1.08 for lanes 1, 2, 3, 4, DENV PAN and ZIKV, respectively.

Statistical Analysis

Origin (OriginLab Corporation) was used to calculate and graph the data. Box and whiskers plots: the black ✕ represent the maximum and minimum measured normalized intensity values, while the black ☐ represents the mean value, and the larger box represents the 25–75% range of the data. Individual colored points represent individual patient samples measured.. Unpaired, two-tailed T-test or one-way ANOVA was performed to test for statistical significance, as indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Acknowledgments:

References and Notes

1. Ong A, Sandar M, Chen MI, Sin LY, Fatal dengue hemorrhagic fever in adults during a dengue epidemic in Singapore, Int. J. Infect. Dis 11, 263–267 (2007). [PubMed: 16899384]

2. Muller DA, Young PR, The flavivirus NS1 protein: Molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker, Antiviral Res 98, 192–208 (2013). [PubMed: 23523765]

3. Andries A-C, Duong V, Ong S, Ros S, Sakuntabhai A, Horwood P, Dussart P, Buchy P, Evaluation of the performances of six commercial kits designed for dengue NS1 and anti-dengue IgM, IgG and IgA detection in urine and saliva clinical specimens, BMC Infect. Dis 16, 201 (2016). [PubMed: 27184801]

4. Wang SM, Sekaran SD, Early diagnosis of Dengue infection using a commercial Dengue Duo rapid test kit for the detection of NS1, IGM, and IGG, Am. J. Trop. Med. Hyg 83, 690–695 (2010). [PubMed: 20810840]

5. Lee J, Kim H-Y, Chong C-K, Song H-O, Development and clinical evaluation of a highly accurate dengue NS1 rapid test: from the preparation of a soluble NS1 antigen to the construction of an RDT, Diagn. Microbiol. Infect. Dis 82, 128–134 (2015). [PubMed: 25824725]

6. Tricou V, Vu HT, Quynh NV, Nguyen CV, Tran HT, Farrar J, Wills B, Simmons CP, Comparison of two dengue NS1 rapid tests for sensitivity, specificity and relationship to viraemia and antibody responses, BMC Infect. Dis 10, 142 (2010). [PubMed: 20509940]

7. Hang VT, Nguyet NM, Trung DT, Tricou V, Yoksan S, Dung NM, Van Ngoc T, Hien TT, Farrar J, Wills B, Simmons CP, Diagnostic accuracy of NS1 ELISA and lateral flow rapid tests for dengue sensitivity, specificity and relationship to viraemia and antibody responses, PLoS Negl. Trop. Dis 3, e360 (2009). [PubMed: 19156192]

8. Fry SR, Meyer M, Semple MG, Simmons CP, Sekaran SD, Huang JX, McElnea C, Huang C-Y, Valks A, Young PR, Cooper MA, The diagnostic sensitivity of dengue rapid test assays is significantly enhanced by using a combined antigen and antibody testing approach, PLoS Negl. Trop. Dis 5, e1199 (2011). [PubMed: 21713023]

9. Osorio L, Ramirez M, Bonelo A, Villar LA, Parra B, Comparison of the diagnostic accuracy of commercial NS1-based diagnostic tests for early dengue infection, Virol. J 7, 361 (2010). [PubMed: 21134275]

10. Fernanda Estofolete C, Terzian ACB, Parreira R, Esteves A, Hardman L, Greque GV, Rahal P, Nogueira ML, Clinical and laboratory profile of Zika virus infection in dengue suspected patients: A case series, J. Clin. Virol 81, 25–30 (2016/8). [PubMed: 27289428]

11. ZIKV Detect IgM Capture ELISA by InBios International, Inc: FDA Safety Communication - Wait for Confirmatory Test Results Before Making Patient Management Decisions MedWatch The FDA Safety Information and Adverse Event Reporting Program (2016) (available at http://www.fda.gov/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/ucm534538.htm).

12. O'Farrell B, in The Immunoassay Handbook, David Wild John Rhys, Sheehan Chris, Binder Steve, and He Jianwen, Ed. (Elsevier, Ltd., 2013), pp. 89–107.

13. Brown WC, Akey DL, Konwerski JR, Tarrasch JT, Skiniotis G, Kuhn RJ, Smith JL, Extended surface for membrane association in Zika virus NS1 structure, Nat. Struct. Mol. Biol (2016) (available at http://www.nature.com/nsmb/journal/vaop/ncurrent/full/nsmb.3268.html).

14. Akey DL, Brown WC, Dutta S, Konwerski J, Jose J, Jurkiw TJ, DelProposto J, Ogata CM, Skiniotis G, Kuhn RJ, Smith JL, Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system, Science 343, 881–885 (2014). [PubMed: 24505133]

15. Falconar AK, Young PR, Production of dimer-specific and dengue virus group cross-reactive mouse monoclonal antibodies to the dengue 2 virus non-structural glycoprotein NS1, J. Gen. Virol 72 (Pt 4), 961–965 (1991). [PubMed: 1849979]

16. Waggoner JJ, Gresh L, Vargas MJ, Ballesteros G, Tellez Y, Soda KJ, Sahoo MK, Nuñez A, Balmaseda A, Harris E, Pinsky BA, Viremia and Clinical Presentation in Nicaraguan Patients

Infected with Zika Virus, Chikungunya Virus, and Dengue Virus, Clin. Infect. Dis (2016), 10.1093/cid/ciw589.

17. D'Ortenzio E, Matheron S, de Lamballerie X, Hubert B, Piorkowski G, Maquart M, Descamps D, Damond F, Yazdanpanah Y, Leparc-Goffart I, Evidence of Sexual Transmission of Zika Virus, N. Engl. J. Med (2016), 10.1056/NEJMc1604449.

18. Korhonen EM, Huhtamo E, Virtala A-MK, Kantele A, Vapalahti O, Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue, J. Clin. Virol 61, 353–358 (2014). [PubMed: 25242312]

19. Chuansumrit A, Chaiyaratana W, Tangnararatchakit K, Yoksan S, Flamand M, Sakuntabhai A, Dengue nonstructural protein 1 antigen in the urine as a rapid and convenient diagnostic test during the febrile stage in patients with dengue infection, Diagn. Microbiol. Infect. Dis 71, 467–469 (2011). [PubMed: 21996098]

20. Bingham AM, 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 (2016) (available at https://espanol.cdc.gov/enes/mmwr/volumes/65/wr/mm6518e2.htm?s_cid=mm6518e2_w&mobile=nocontent).

21. Muller DA, Depelsenaire ACI, Young PR, Clinical and Laboratory Diagnosis of Dengue Virus Infection, J. Infect. Dis 215, S89–S95 (2017). [PubMed: 28403441]

22. Tricou V, Minh NN, Farrar J, Tran HT, Simmons CP, Kinetics of viremia and NS1 antigenemia are shaped by immune status and virus serotype in adults with dengue, PLoS Negl. Trop. Dis 5, e1309 (2011). [PubMed: 21909448]

23. Hirsch AJ, Smith JL, Haese NN, Broeckel RM, Parkins CJ, Kreklywich C, DeFilippis VR, Denton M, Smith PP, Messer WB, Colgin LMA, Ducore RM, Grigsby PL, Hennebold JD, Swanson T, Legasse AW, Axthelm MK, MacAllister R, Wiley CA, Nelson JA, Streblow DN, Zika Virus infection of rhesus macaques leads to viral persistence in multiple tissues, PLoS Pathog 13, e1006219 (2017). [PubMed: 28278237]

24. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, Drake JM, Brownstein JS, Hoen AG, Sankoh O, Myers MF, George DB, Jaenisch T, Wint GR, Simmons CP, Scott TW, Farrar JJ, Hay SI, The global distribution and burden of dengue, Nature 496, 504–507 (2013). [PubMed: 23563266]

25. Kettler H, White K, Hawkes S, in WHO/TDR, (World Health Organization, Geneva, 2004).

26. Peeling RW, Mabey D, Point-of-care tests for diagnosing infections in the developing world, Clin. Microbiol. Infect (2010) (available at http://onlinelibrary.wiley.com/doi/10.1111/j.1469-0691.2010.03279.x/full).

27. Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, Scott RM, Burke DS, Hoke CH, Innis BL, Vaughn DW, Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999, Am. J. Trop. Med. Hyg 68, 191–202 (2003). [PubMed: 12641411]

28. Forshey BM, Reiner RC, Olkowski S, Morrison AC, Espinoza A, Long KC, Vilcarromero S, Casanova W, Wearing HJ, Halsey ES, Kochel TJ, Scott TW, Stoddard ST, Incomplete Protection against Dengue Virus Type 2 Re-infection in Peru, PLoS Negl. Trop. Dis 10, e0004398 (2016). [PubMed: 26848841]

29. Villar L, Dayan GH, Arredondo-García JL, Rivera DM, Cunha R, Deseda C, Reynales H, Costa MS, Morales-Ramírez JO, Carrasquilla G, Rey LC, Dietze R, Luz K, Rivas E, Miranda Montoya MC, Cortés Supelano M, Zambrano B, Langevin E, Boaz M, Tornieporth N, Saville M, Noriega F, CYD15 Study Group, Efficacy of a tetravalent dengue vaccine in children in Latin America, N. Engl. J. Med 372, 113–123 (2015). [PubMed: 25365753]

30. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB, Phylogeny of the genus Flavivirus, J. Virol 72, 73–83 (1998). [PubMed: 9420202]

31. Saito Y, Moi ML, Kotaki A, Ikeda M, Tajima S, Shiba H, Hosono K, Saijo M, Kurane I, Takasaki T, Detecting Dengue Virus Nonstructural Protein 1 (NS1) in Urine Samples Using ELISA for the Diagnosis of Dengue Virus Infection, Jpn. J. Infect. Dis 68, 455–460 (2015). [PubMed: 25766601]

32. Abramoff MD, Magelhaes PJ, Ram SJ, Image Processing with ImageJ, Biophotonics International 11, 36–42 (2004).

33. Tyrrell CH, A Lateral Flow Smart Phone Image Analysis Diagnostic, 10.15368/theses.2013.165.

34. Peeling RW, Artsob H, Pelegrino JL, Buchy P, Cardosa MJ, Devi S, Enria DA, Farrar J, Gubler DJ, Guzman MG, Halstead SB, Hunsperger E, Kliks S, Margolis HS, Nathanson CM, Nguyen VC, Rizzo N, Vázquez S, Yoksan S, Evaluation of diagnostic tests: dengue, Nat. Rev. Microbiol 8, S30–8 (2010). [PubMed: 21548185]

35. Miller RK, Zeuch N, Machine Vision (Springer Science & Business Media, 1989).

36. Andries AC, Duong V, Ngan C, Ong S, Huy R, Sroin KK, Te V, B. Y, Try PL, Buchy P, Field evaluation and impact on clinical management of a rapid diagnostic kit that detects dengue NS1, IgM and IgG, PLoS Negl. Trop. Dis 6, e1993 (2012). [PubMed: 23301110]

37. Andries A-C, Duong V, Ly S, Cappelle J, Kim KS, Lorn Try P, Ros S, Ong S, Huy R, Horwood P, Flamand M, Sakuntabhai A, Tarantola A, Buchy P, Value of Routine Dengue Diagnostic Tests in Urine and Saliva Specimens, PLoS Negl. Trop. Dis 9, e0004100 (2015). [PubMed: 26406240]

38. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, Paris DH, Premaratna R, de Silva HJ, Lalloo DG, Day NP, Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy, Clin. Vaccine Immunol 18, 2095–2101 (2011). [PubMed: 22012979]

39. Bhamla MS, Benson B, Chai C, Katsikis G, Johri A, Prakash M, Hand-powered ultralow-cost paper centrifuge, Nature Biomedical Engineering 1, 0009 (2017).

40. Vivek R, Ahamed SF, Kotabagi S, Chandele A, Khanna I, Khanna N, Nayak K, Dias M, Kaja M-K, Shet A, Evaluation of a pan-serotype point-of-care rapid diagnostic assay for accurate detection of acute dengue infection, Diagn. Microbiol. Infect. Dis 87, 229–234 (2017). [PubMed: 27955870]

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

42. Pardee K, Green AA, Takahashi MK, Braff D, Lambert G, Lee JW, Ferrante T, Ma D, Donghia N, Fan M, Daringer NM, Bosch I, Dudley DM, O'Connor DH, Gehrke L, Collins JJ, Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components, Cell 165, 1255–1266 (2016). [PubMed: 27160350]

43. Lanciotti RS, Calisher CH, Gubler DJ, Chang GJ, Vorndam AV, Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction, J. Clin. Microbiol 30, 545–551 (1992). [PubMed: 1372617]

44. Becerra A, Warke RV, Xhaja K, Evans B, Evans J, Martin K, de Bosch N, Rothman AL, Bosch I, Increased activity of indoleamine 2,3-dioxygenase in serum from acutely infected dengue patients linked to gamma interferon antiviral function, J. Gen. Virol 90, 810–817 (2009). [PubMed: 19264674]

45. Becerra A, Warke RV, Martin K, Xhaja K, de Bosch N, Rothman AL, Bosch I, Gene expression profiling of dengue infected human primary cells identifies secreted mediators in vivo, J. Med. Virol 81, 1403–1411 (2009). [PubMed: 19551822]

46. Lukman N, Salim G, Kosasih H, Susanto NH, Parwati I, Fitri S, Alisjahbana B, Widjaja S, Williams M, Comparison of the Hemagglutination Inhibition Test and IgG ELISA in Categorizing Primary and Secondary Dengue Infections Based on the Plaque Reduction Neutralization Test, Biomed Res. Int 2016, 5253842 (2016). [PubMed: 27446953]

47. King CC, Chao DY, Wu HL, Chang GJ, Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health, J. Microbiol. Immunol. Infect (2005) (available at http://www.academia.edu/download/44310012/46.pdf).

48. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang A, Miller W, Lipmann DJ, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res 25, 3389–3402 (1997). [PubMed: 9254694]
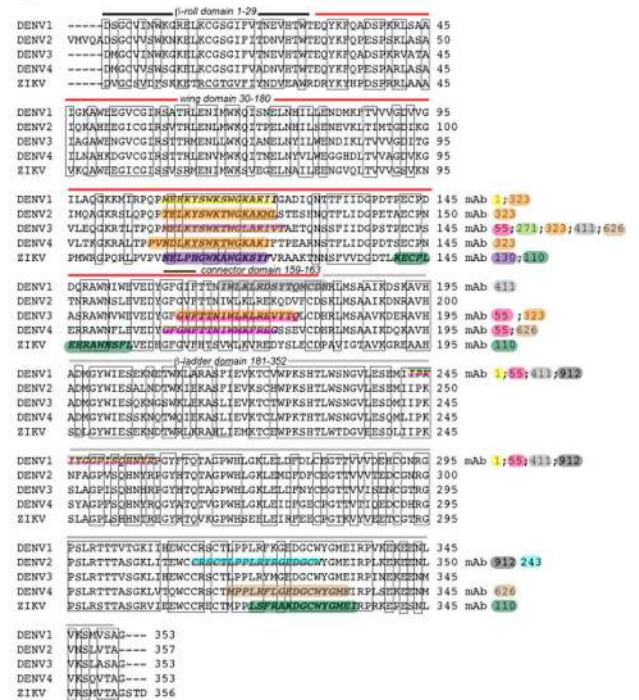
Fig. 1. NS1 protein alignment and linear epitope mapping of the ten antibodies used to run the dengue virus serotype-specific NS1 rapid tests, the pan-dengue NS1 test, and the Zika virus NS1 test.

(**A**) Table listing mAb names, mAb immunochromatography applications, mAb linear epitope sequences and starting amino acid positions, and NS1 domain positions. (**B**) Comparison of amino acid similarity based on analysis of NS1 protein sequences from the following viruses: DENV1- Strain Singapore/S275/1990, accession number P33478; DENV2 -Strain NGC, accession number AAA42941; DENV3- Philippines/H87/1956, accession number AAA99437; DENV4- Singapore/8976/1995, accession number AAV31422; Zika virus, accession number KU497555.1. Amino acid sequences were compared using Color Align Conservation http://www.bioinformatics.org/sms2/color_align_cons.html to enhance the output of sequence alignment program. Residues that are identical among the sequences are boxed. Linear peptide epitopes (B) are italicized and indicated in color on the figure, with the key to the right of the figure.
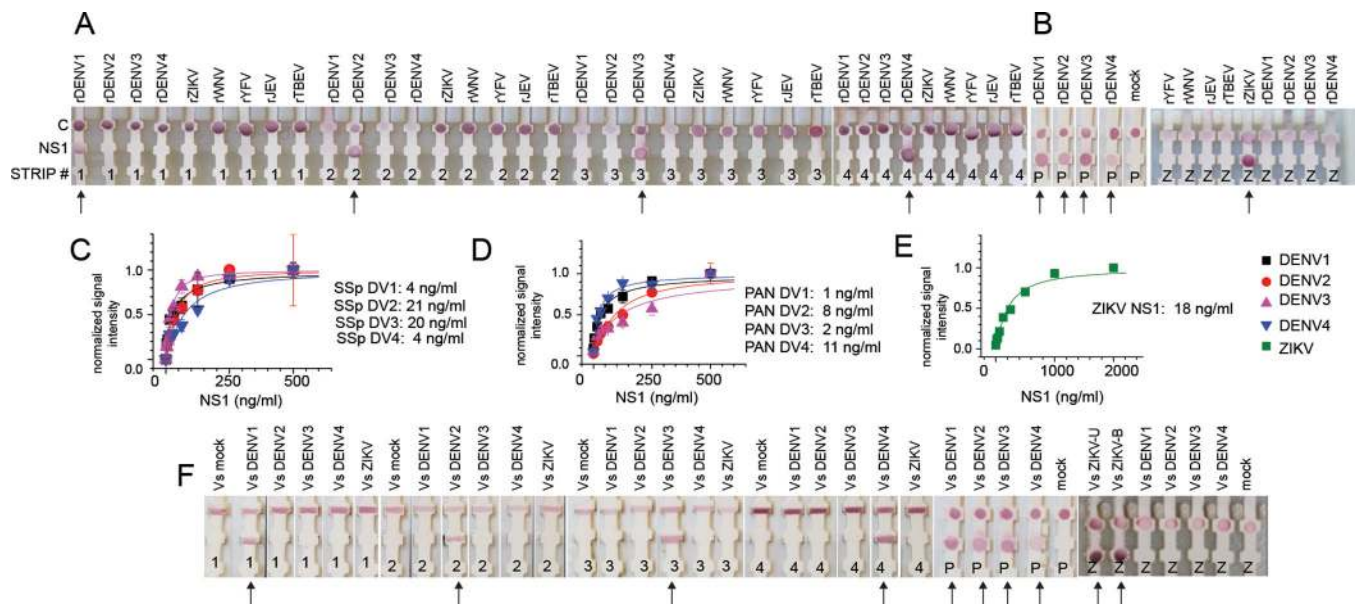
Fig. 2. Rapid immunochromatography for specific detection of DENV NS1 proteins (serotypes 1–4) and ZIKV NS1 protein.

(A-B) Images of rapid test strips. Strip numbers refer to the DENV serotype NS1 (1–4), pan-dengue (P; all four DENV serotype NS1 proteins), or ZIKV virus NS1 (Z) detected. Recombinant NS1 proteins, indicated with a lower case "r' preceding the virus name, were prepared at 500 ng/ml, and the strips were run using 50 µl of solution. Strip #1 (detects DENV serotype 1): mAb pair 912/271; Strip #2 (detects DENV serotype 2): mAb pair 243/1; Strip #3 (detects DENV serotype 3): mAb pair 411/55; Strip #4 (detects DENV serotype 4): mAb pair 626/55; Strip P ("pan dengue"; detects all four DENV serotypes): mAbs 271–243-411–626/323; Strip Z (detects ZIKV): mAbs 130/110. The test proteins run on the strips are recombinant DENV NS1, serotypes 1–4 (rDENV1-rDENV4), as well as recombinant NS1 proteins from ZIKV (rZIKV), West Nile Virus (rWNV), Yellow Fever Virus (rYFV), Japanese encephalitis virus (rJEV), and Tick Borne encephalitis virus (rTBEV). C = control, NS1 = detection site for specific NS1 protein. (C-E) Limits of detection for viral NS1 proteins using the serotype-specific (SSp) DENV strips 1–4 (C), the pan-dengue strip (D), and the ZIKV virus strip (E). The limits of detection, representing three independent determinations, are recorded on the figures. Each point (C-E) is presented as the mean and standard deviation. (F) NS1-containing supernatants from Vero cells infected with DENV serotypes 1–4 (Vs DENV1–4) or ZIKV virus (Vs ZIKV-U (Uganda) or ZIKV-B (Brazil)) were chromatographed on strips 1–4, pan-dengue (P), or ZIKV (Z) NS1 strips. The arrows indicate the strips with positive NS1 signals. Horizontal test lines (panel F) result from applying antibodies to the nitrocellulose using a mechanical striper device; the circular dot signals result from applying antibodies to the nitrocellulose using a standard pipettor.
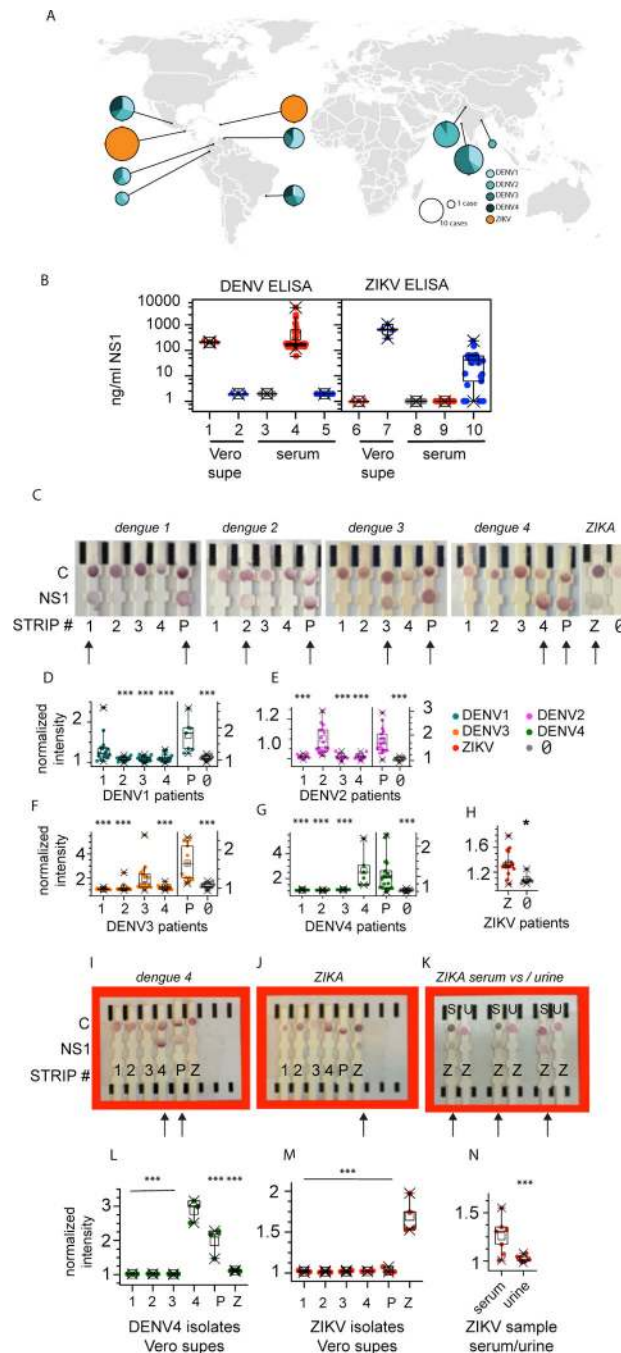
Fig. 3. Applying the rapid test to analyze human patient sera.
(**A**) Map showing the endemic virus regions where the rapid tests were deployed to analyze
patient serum samples. The areas of the circles correlate with the numbers of samples
analyzed. The blue colors, faint to dark, represent DENV serotypes 1–4. ZIKV is indicated
in orange color. (**B**) ELISA results showing the amounts of DENV (left) and ZIKV (right)
NS1 found in patient serum and supernatants from infected cell cultures. Lanes 1 and 6 are
supernatants from Vero cells infected with DENV; lanes 2 and 7 are supernatants from Vero
cells infected with ZIKV. Lanes 3 and 8 are PCR-negative sera; lanes 4 and 9 are sera from

PCR-positive DENV patients. Lanes 5 and 10 are sera from PCR-positive ZIKV patients. (**C**) Images of rapid test analysis of DENV NS1 serotypes 1–4 and ZIKV NS1 on serotype specific strips 1–4, as well as pan-dengue (P) and ZIKV (Z); the upward arrows mark positive tests, and θ is serum from an uninfected patient. (**D-G**) Quantification of rapid test results. Dipstick tests were run with PCR-confirmed DENV sera or ELISA-validated ZIKV serum (panel C), and the resulting signals were quantified and expressed as box plots. Statistical significance, based on one one-way ANOVA, is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. (**H**) Statistical significance, based on an unpaired T-test, is presented as * $P < 0.05$. Box and whiskers plots: the black ✕ represent the maximum and minimum measured normalized intensity values, while the small square box ☐ represents the mean value, and the larger box represents the 25–75% range of the data. Individual colored points represented individual patient samples measured. (**I-J**) Images of rapid tests showing that DENV and ZIKV NS1 tests do not cross-react. (**I**) Supernatants from Vero cells infected with DENV serotype 4 were chromatographed on DENV serotype strips 1–4, on the pan-dengue strip (P), and on the ZIKV NS1 strip (Z). (J) Supernatants from Vero cells infected with ZIKV virus were chromatographed on DENV serotype strips 1–4, on the pan-dengue strip (P), and on the ZIKV NS1 strip (Z). (**K**) Images of rapid tests showing ZIKV NS1 is detected in serum samples concentrated 5X, but ZIKV virus NS1 is not detected in concentrated urine. Three sets of paired serum and urine samples were concentrated 5X by filter centrifugation and chromatographed on the ZIKV dipsticks. S: serum; U: urine. (**I-K**) The red boxes and vertical black lines serve as fiducial markers for image recognition and processing. Upward arrows indicate positive tests, using the serum samples. (**L-N**) Quantification of NS1 protein in supernatants of Vero cells infected separately with three DENV4 patient isolates (L) or three ZIKV patient isolates (M), or five paired serum/urine patient samples (N). Fig. 3L-N: One-way ANOVA was used to calculate statistical significance of the dengue and Zika tests: p<0.05, p<0.01, and p<.0001 are indicated as *, **, and ***, respectively. Box and whiskers plots: the black ✕ represent the maximum and minimum measured normalized intensity values, while the black ☐ represents the mean value, and the larger box represents the 25–75% range of the data. Individual colored points represented individual patient samples measured.
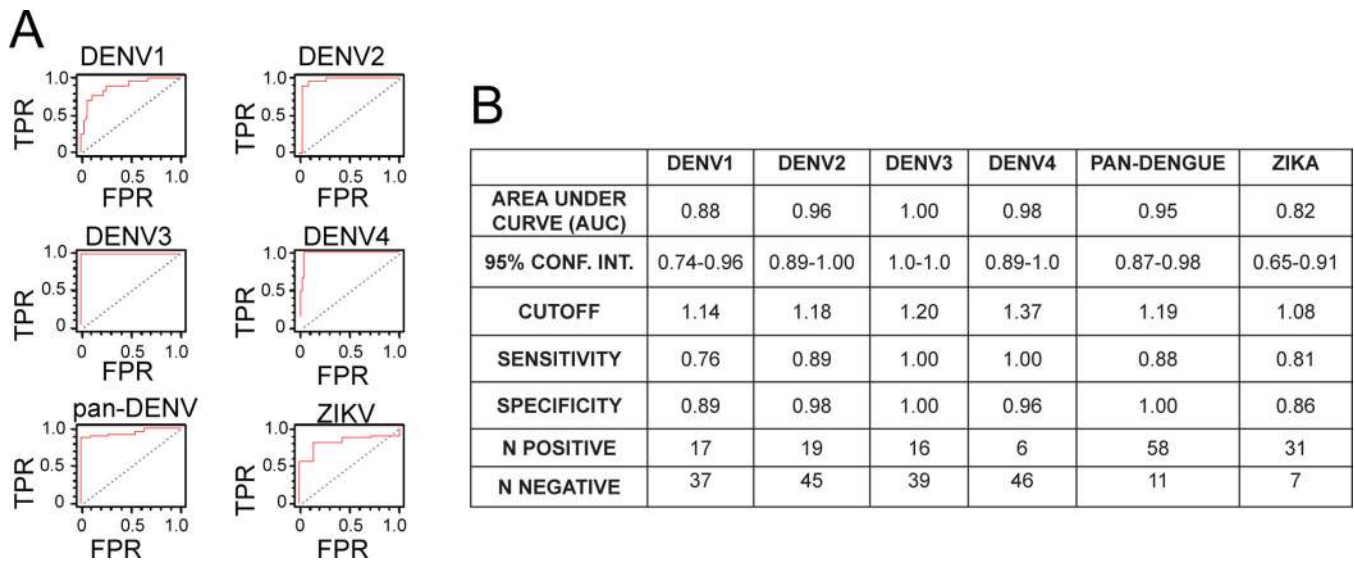
Fig. 4. ROC analysis, sensitivity/specificity analysis, and 95% confidence intervals (95% conf. int.) of the dengue and Zika tests.

(**A**) ROC curve analysis of the patient sample data collected for dengue serotypes 1–4, Dengue Pan, and Zika virus. TPR: true positive rate (sensitivity); FPR: false positive rate (1-specificity). (**B**) Table listing numerical values of the sensitivity and specificity results.

	DENV1	DENV2	DENV3	DENV4	PAN-DENGUE	ZIKA
AREA UNDER CURVE (AUC)	0.88	0.96	1.00	0.98	0.95	0.82
95% CONF. INT.	0.74-0.96	0.89-1.00	1.0-1.0	0.89-1.0	0.87-0.98	0.65-0.91
CUTOFF	1.14	1.18	1.20	1.37	1.19	1.08
SENSITIVITY	0.76	0.89	1.00	1.00	0.88	0.81
SPECIFICITY	0.89	0.98	1.00	0.96	1.00	0.86
N POSITIVE	17	19	16	6	58	31
N NEGATIVE	37	45	39	46	11	7