Competitive ELISA for a serologic test to detect dengue serotype-specific anti-NS1 IgGs using high-affinity UB-DNA aptamers

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Research Article

Keywords: Viral Infections, Aptamer-antibody Sandwich System, DEN-NS1, Clinical Blood Samples, Secondary Infections

Posted Date: April 21st, 2021

DOI: https://doi.org/10.21203/rs.3.rs-428449/v1

Abstract

Serologic tests to detect IgGs specific to antigens related to viral infections are urgently needed for diagnostics and therapeutics in endemic, epidemic, and pandemic situations. We present a diagnostic method for serotype-specific IgG identification of dengue infection by a competitive enzyme-linked immunosorbent assay (ELISA), using high-affinity unnatural-base-containing DNA (UB-DNA) aptamers. Dengue is a widespread mosquito-borne viral disease, with four categorized serotypes. Using UB-DNA aptamers that bind specifically to each serotype or sub-serotype dengue NS1 protein (DEN-NS1), we developed an ELISA format with an aptamer – antibody sandwich system for dengue diagnostics. We found that IgGs highly specific to DEN-NS1 inhibit the serotype-specific NS1 detection by the ELISA format, inspiring us to develop another competitive ELISA format for serotype-specific IgG detection of dengue infection. Analyses of clinical blood samples from Singaporean patients with primary or secondary infections confirmed the highly specific IgG detection of this format, and the IgG production initially reflected the serotype of the past infection, rather than that of the recent infection. The combination of the ELISA and competitive ELISA using the UB-DNA aptamers allows the diagnosis of both past and current viral infections and will facilitate prompt and specific medical care and vaccine development for infectious diseases.

Introduction

The recent COVID-19 (Coronavirus Disease 2019) pandemic has highlighted the importance of serologic tests, for infectious disease diagnostics complementary to PCR and biomarker-detection tests[1-4]. Serologic tests enable the detection of viral-specific antibodies, mainly IgM and IgG, produced in the body by responses to current and past infections. In addition, such tests are useful for the diagnoses of infection, including surveys of disease transmission, infection spread, and acquired immunity, as well as evaluations of vaccine development. Most typical serologic tests are methods to detect antibodies by binding to viral-related antigens using point-of-care tests, such as lateral flow devices and the enzyme-linked immunosorbent assay (ELISA) or chemiluminescent immunoassay (CIA) format[5-8]. In these methods, the sensitivity and specificity of detection due to the cross-reactivity with other related diseases are one of the key issues, as they cause false-negative and false-positive test results. Indeed, diseases such as COVID-19 and dengue virus (DENV) infection present with similar symptoms in the tropical and subtropical countries[9,10].

We now report a novel serologic test by a competitive ELISA format, using high-affinity unnatural-base-containing DNA (UB-DNA) aptamers for dengue diagnostics. Dengue is an arthropod-borne flavivirus with four main serological types (DEN1–4). Worldwide DENV infections have been estimated at more than 390 million annually, and the recent dramatic and global increase in incidence suggests that approximately 40% of the world's population is now at risk[11]. The symptoms range from mild in most cases to severe and occasionally fatal[12]. Infection with one DENV serotype provides long-term protection from re-infection with the same serotype, but may enhance the disease from a secondary heterotypic

infection. A secondary DENV infection with a different serotype from the primary infection is the greatest risk factor for severe diseases, such as Dengue Haemorrhagic Fever and Dengue Shock Syndrome. Antibody-dependent enhancement (ADE) is thought to be one of the mechanisms responsible for severe dengue[13-19]. The immune history is important for understanding subsequent disease risk and protection[20-27], and thus the ability to identify the previous infected serotype is invaluable to study the pathogenesis. Quantitative diagnostic methods for serotype identification in previously infected patients will allow us to elucidate whether the sequence of the dengue serotype infection affects the severity of the disease.

Currently, there is no specific treatment for dengue, but early diagnostics prompt proper medical attention and reduce the risk of fatality. Dengvaxia, a dengue vaccine, was developed by Sanofi Pasteur[28,29]. However, analyses revealed that the vaccination of persons who have never been infected with dengue led to a higher risk of more severe symptoms when they became infected after the vaccination[30-34]. Consequently, the World Health Organization (WHO) advised the use of the vaccine only in people previously infected with dengue. The effectiveness of the vaccine could be different for people previously infected with different serotypes of dengue, and more research is needed to understand the mechanisms. Therefore, the development of detection methods for not only current infection but also past infection, including the serotype identification and quantification, is an urgent worldwide task[31,34].

In the early stage of DENV infection (within one week after fever onset, Figure 1a), the viral RNA can be identified by RT-qPCR. Virus-related materials, such as the envelope protein and non-structural protein 1 (NS1), can also be detected by ELISA or lateral flow assay (LFA), using antibodies to the antigens. RT-qPCR is useful for serotype identification in the early stage, but not in the later stage[8,35,36]. For the serotype-specific NS1 detection in the early stage, several reports have described the generation of antibodies to each NS1 serotype[37-40], but their diagnostic kits are not commercially available. In the later stage, the patients' IgM and IgG antibodies to viral-related antigens, such as viral particles and NS1 proteins, are detectable by ELISA or LFA. The IgG production continues throughout life, and thus IgG detection enables the identification of past infections. However, the detection reliabilities are still limited, and the serotype identification remains difficult. Furthermore, the cross-reactivity of the tests with other infectious diseases showing similar symptoms, such as COVID-19 and other flavivirus infections, is a serious problem[9,10,41,42]. In some COVID-19 patients in Singapore, a dengue serologic test kit revealed false positives for DENV infection[10].

Here, we present a simple and highly sensitive serotype-specific detection method for the anti-DEN-NS1 IgG antibodies in the early and later stages of DENV infection, as well as for the NS1 proteins in the early stage, by ELISA with high-affinity DNA aptamers (Figure 1b and 1c). DNA aptamers are single-stranded DNA fragments that bind specifically to target molecules [43,44], and are considered to serve as antibody alternatives. We developed a method (ExSELEX, genetic alphabet Expansion for Systematic Evolution of Ligands by EXponential enrichment) using an unnatural base pair (UBP), Ds–Px (Figure 2a), to generate DNA aptamers with increased affinities to targets, in which unnatural base (UB) components, such as Ds

and Px/Pa, are introduced into DNA aptamers as fifth and sixth letters[45-48]. Using ExSELEX, we generated a series of UB-DNA aptamers targeting each serotype or sub-serotype of DEN-NS1 proteins (Figure 2b)[49]. The ELISA format using a sandwich system with these UB-DNA aptamers and an anti-DEN-NS1 antibody detects and identifies the serotype or sub-serotype of DEN-NS1 in clinical blood samples with extremely high specificity (Figure 1b)[49]. During the studies, we serendipitously found that the UB-DNA aptamer binding to DEN-NS1 is serotype-specifically inhibited when anti-DEN-NS1 IgG antibodies were present in patient blood samples. Based on this observation, we developed a competitive ELISA format for a serological test to quantitatively detect serotype-specific IgGs for dengue diagnostics (Figure 1c). Using this method, we analyzed clinical samples from dengue patients. The tests with the secondary infection samples revealed that the IgG production reacted more to the serotype of the past infection, rather than that of the secondary infection. This dengue diagnostic system using the competitive ELISA format will provide valuable information about dengue epidemiology and pathogenesis, as well as vaccine usage[31,34] and development[50,51], and could be adapted for other diseases.

Results

UB-DNA aptamers targeting each DEN-NS1 serotype and the ELISA format. We previously developed an ELISA format of aptamer–antibody sandwich systems (Figure 1b), using UB-DNA aptamers targeting each serotype or sub-serotype of DEN-NS1 (Figure 2), for diagnostics in the early stage of dengue infection (Figure 1a)[49]. These high-affinity UB-DNA aptamers (K_D = 30–182 pM) were generated from five-letter Ds-DNA libraries by ExSELEX, involving the Ds–Px pair as a third base pair in PCR. In the aptamer generation, we used each DEN-NS1 serotype purchased from The Native Antigen Company. Three aptamers, AptD1, AptD3, and AptD4 targeting DEN1-NS1, DEN3-NS1, and DEN4-NS1, respectively, contained two Ds bases, while the isolated aptamer, AptD2, exhibiting the highest affinity to DEN2-NS1, contained two Ds and one Px bases. These three UBs in AptD2 are essential for the tight binding to DEN2-NS1. This Px base was incorporated into the aptamer by a mutation during PCR amplification of the DNA libraries in ExSELEX. Since the Px nucleoside is unstable and thus not suitable for chemical DNA synthesis, instead of the Px nucleoside, we used the Pa nucleoside, in which the nitro group is replaced with the aldehyde group (Figure 2a)[49,52,53], for the chemical synthesis of AptD2. Each of these four aptamers contains an extraordinarily stable mini-hairpin sequence[54-56] at the 3'-terminus, in which the loop region is useful as a modification site with biotin for aptamer immobilization[48,57-59].

For the ELISA format with aptamer and antibody sandwich systems, we employed an anti-DEN-NS1 monoclonal antibody (Ab#D06), which binds to all four serotypes of DEN-NS1 with 27–107 pM K_D values[49]. In the ELISA format, the mixtures of DEN-NS1 samples and the antibody Ab#D06 were incubated on an aptamer-immobilized plate, and then the signal was detected by the colorimetric output, using a secondary anti-IgG HRP-conjugated antibody. We determined the limits of detection (LOD) in buffer, which were 1.19–2.36 ng/mL for the DEN-NS1 samples purchased from The Native Antigen Company[49].

Development of serotype-specific anti-DEN-NS1 IgG detection by a competitive ELISA format. We next tested this ELISA format in the presence of human serum purchased from Sigma-Aldrich, for the direct detection of each DEN-NS1 from The Native Antigen Company (Figure 3). Unexpectedly, we found that the NS1 detection was significantly inhibited in the serum (Figure 3b), relative to that in buffer (Figure 3a). One of the plausible causes is the presence of anti-DEN-NS1 IgG antibodies in the serum, which inhibited the binding of the aptamer to the additional NS1 proteins. To validate this IgG contamination theory, we removed the total IgG antibodies from the serum by treating it with protein A-immobilized resin, and confirmed the absence of inhibition with the treated serum (Figure 3c). We also performed an ELISA using a serum sample from a Singaporean who was currently not infected with dengue, to determine whether the serum inhibited the detection. Interestingly, the serum showed the serotype-specific inhibitions in the detection of DEN2-NS1, as well as DEN1-NS1 to some extent (Figure 3d), suggesting that the person might have previously been infected with the dengue serotype 2 and/or serotype 1 viruses. Therefore, we obtained two other serum samples from volunteers from a dengue non-endemic country, and performed an ELISA. As expected, the two serum samples did not inhibit the DEN-NS1 detection in our ELISA format (Figure 3e and 3f). For further studies, we used these serum samples as a control without anti-DEN-NS1 IgGs. These results inspired us to develop a new method using the competitive ELISA format with each DEN-NS1 serotype sandwiched with each aptamer and Ab#D06, for the serotype-specific detection of anti-DEN-NS1 IgG antibodies in human serum samples (Figure 1c).

For the serotype-specific IgG detection, we developed a simple quantification method for the anti-DEN-NS1 IgG activities (Supplementary Figure 2). To this end, we performed competitive-inhibition in ELISA using a series of different volumes (0.05, 0.1, 0.2, 0.5, and 5 μL) of patient serum, in the presence of a certain amount of each DEN-NS1 serotype (The Native Antigen Company). After the absorbance measurement at 450 nm (OD_{450}) in the ELISA format, the OD_{450} values were plotted against the volume of serum, and we calculated the serum volume required to give an OD_{450} of 1.0. We then defined the relative IgG activity (Activity), by the following formula: Activity = 5 / (the serum volume required for an OD_{450} of 1.0). To equalize the sensitivities of the DEN-NS1 detection among the four serotypes, we adjusted the amounts of immobilized aptamers and spiked DEN-NS1 for each serotype (Supplementary Figure S1).

Serotype-specific detection of anti-DEN-NS1 IgG antibodies in patient samples. Using this competitive ELISA format and the IgG quantification method, we measured the longitudinal changes in the IgG production and the serotype specificities of the archived blood samples from eleven Singaporean patients (PD1-1 to PD4-1) with acute DENV infection (Figure 4). The dengue serotype of the current infection in each sample was also identified by an FTD dengue differentiation RT-qPCR test from Fast Track Diagnostics and by sequencing the RT-PCR amplicons of the clinical samples obtained within 3—5 days after fever onset. In addition, we performed the DEN-NS1, IgM, and IgG detections in these samples with the commercially available LFA kits from SD BIOLINE for DEN-NS1 detection and from Panbio for IgM/IgG detection.

Our competitive ELISA format successfully detected the IgGs even after one year in the recovered patients, as shown in the samples PD2-3, PD3-1, and PD3-3. The samples can be categorized into two groups by the IgG detection: one group included PD1-1, PD1-2, PD1-3, PD2-1, PD2-2, PD3-1, and PD3-2, in which the IgG was not detected within a week after fever onset, and the other group included PD2-3, PD3-3, PD3-4, and PD4-1, in which the IgG was detected within 3–5 days after fever onset. These data suggested that the latter patients were previously infected by dengue (Figure 1a). Thus, the first group most likely represented the primary infection, and the second group was a secondary or higher infection.

There were some discrepancies in PD1-1, PD3-2, and PD3-3 between our IgG detection and the conventional LFA method for IgM/IgG detection. The visual judgement using the LFA format was often ambiguous, and all of the longitudinal IgG detection data supported the higher accuracy of our method over that of the LFA format. The sensitivity and specificity of the LFA kit reported in the literature are 71.9% and 95%, respectively.[8] Thus, our IgG detection might faithfully identify the primary or secondary infection of patients within 3–5 days after fever onset.

Our data indicate that the inhibition of the aptamer binding to each DEN-NS1 serotype resulted from the anti-NS1 IgG, rather than the anti-NS1 IgM. In the samples from the patients with the primary infection, IgM was detected in PD1-1, PD2-1, PD3-1, and PD3-2 by the LFA (Figure 4). In contrast, no inhibition of the DEN-NS1 detection in our competitive ELISA occurred within the primary infection samples in the first week after fever onset, and the inhibition became detectable at 17 days or thereafter in longitudinal studies. Thus, the IgMs might not inhibit the UB-DNA aptamer binding to DEN-NS1, and our competitive ELISA format specifically detected only IgGs.

Using the four samples (PD1-2, PD1-3, PD2-2, and PD3-1) collected 17 or more days after fever onset in the primary infection, our competitive ELISA format identified the serotype of the current infection from the highest serotype-specific activity of each detected IgG. Our results were identical with those obtained by RT-qPCR and sequencing. In the samples obtained six months later (PD1-2 and PD2-2), our data indicated that the serotype-specificity of IgGs broadened to other serotypes.

In the secondary infection samples (PD2-3, PD3-3, PD3-4, and PD4-1), the initial IgG level was not identical to the serotype-specificity of IgGs in the current infection, which might mainly reflect the serotypes of the past infection[26,27,60]. Even after one week, the production of the IgG antibodies that predominantly recognized the serotype resulting from the presumable past infection increased sharply, as compared to the IgGs produced from the current secondary infection. Although the predominance of the past infection varied depending on the patients, the PD2-3 and PD3-3 patient samples revealed the massive production of the IgG antibodies to the past serotype infection, and thus the competitive ELISA format might identify the dengue serotype of the past infection.

Comparison with the typical conventional serologic test. To evaluate the competitive ELISA format, we compared the specificity and sensitivity of the format with a conventional serologic test reported in the literature[50,61]. There are several available kits for the direct DENV IgM and IgG detections by ELISA

formats, which cannot identify the serotype, and their sensitivities and specificities for IgG detection are 45–56% and 93–95%, respectively, with Panbio Dengue Virus IgG Capture ELISA, and 55–89% and 64–99% with SD BIOLINE Dengue IgG ELISA[8,62,63]. Thus, we performed the conventional serologic test by incubating clinical samples on the plate immobilized with each serotype DEN-NS1 (The Native Antigen Company), followed by detection with a secondary anti-IgG HRP-conjugated antibody (Figure 1d).

First, we compared the inhibition specificity by the competitive ELISA (Figure 5a) and the IgG detection by the conventional method (Figure 5b), using various volumes (0.05–5 μL) of a Singaporean clinical plasma sample, PD2-4. Although the IgGs in the sample were not detected by the LFA kit, our method and the conventional methods clearly detected anti-DEN-NS1 IgGs. In our competitive ELISA, the aptamer binding to serotype 3 of DEN-NS1 (DEN3-NS1) was mainly inhibited with the clinical sample (0.1–5 μL of PD2-4), indicating that the IgGs in the sample specifically bind to DEN3-NS1. However, in the conventional method, the IgGs equally bound to all four DEN-NS1 serotypes and the serotype-specificity of the IgGs was unobservable.

Next, we extensively tested the IgG detections using 23 Singaporean clinical samples (PD1-1 to PD4-1) obtained within 3–5 days after fever onset (Supplementary Table 2). The serotypes of the current infection of each sample were initially determined by RT-qPCR and sequencing, and the IgM and IgG detections were also performed with the LFA Panbio kit. The LFA tests detected anti-DEN-NS1 IgGs in the PD1-1, 1-9, 1-14, 1-19, 2-3, 3-2, 3-4, and 4-1 samples, suggesting that these patients had secondary infections. However, there are some discrepancies (including PD2-4, as shown in Figure 5) among the results obtained using the LFA Panbio kit and our conventional and competitive ELISA methods (Figure 6). The conventional and/or competitive ELISA methods detected IgGs in the samples of PD1-5, 1-10, 1-11, 1-18, 2-4, and 3-2, which were not detected by the LFA Panbio kit. In addition, the PD1-1, 1-10, 1-18, and 3-2 samples were IgG-positive by the conventional method, but the competitive ELISA detected no IgGs in these samples. Therefore, further extensive research is necessary to assess the sensitivity and specificity of our method by comparisons to those of the conventional method.

Comparison of the competitive ELISA formats between aptamer–antibody and antibody–antibody sandwich systems. Competitive or blocking ELISA formats using the inhibition between protein–protein or protein–antibody interactions are well-known methods[6,64,65]. Theoretically, the DENV-serotype-specific IgG detection by the competitive ELISA format is also possible by using an antibody–antibody sandwich system, even if these antibodies have no specificity to each DEN-NS1 serotype (Figure 7a). We tested this antibody–antibody system using Ab#D06 and Ab#D25, which do not compete with each other for the DEN-NS1 binding, and compared the longitudinal changes of the IgG detections with the aptamer–antibody system in the PD1-3, 2-2, 2-3, 3-4, and 4-1 samples (Figure 7 and Supplementary Figure 3–6). The anti-DEN-NS1 IgG in the patient serum also inhibited the DEN-NS1 ternary complex formation with the antibody—antibody sandwich pair, and the test exhibited similar patterns to those obtained by the aptamer—antibody sandwich pair. However, the antibody—antibody pair could not detect the IgG activities in the early stage of infection, such as the day 5 sample of PD2-3 and the day 3 sample of PD4-

1. Thus, the sensitivity of the aptamer–antibody system is superior to than that of the antibody–antibody system.

Discussion

We have presented serotype-specific detection methods as a serologic test for IgG antibodies to DEN-NS1 in human blood samples, by the competitive ELISA format using high-affinity UB-DNA aptamers. Only IgGs with strong affinity to each of DEN-NS1 serotypes inhibit the DEN-NS1 detection by the UB-DNA aptamer–antibody sandwich system, allowing for the serotype-specific IgG detection. This IgG detection provides valuable information for the dengue diagnostics and the development and use of the dengue vaccine. (1) The competitive ELISA format can identify the secondary infection within several days (during the febrile period) after fever onset. If anti-DEN-NS1 IgG antibodies are detected in patients within one week after fever onset, then this indicates a secondary infection that may warrant close monitoring. (2) The format can identify the serotypes in the late stage of the primary DENV infection and in the early stage of the secondary infection, in which the serotypes of the past infection can be determined. In combination with the ELISA format using the UB-DNA aptamer–antibody sandwich system, the serotypes of both the current and past infections, as well as the infection status, could be determined in the early stage of DENV infection, enabling prompt and specific medical treatment. (3) The format can check whether a person has been previously infected with DENV and determine the serotype, which is useful for personalized healthcare. (4) The format could provide valuable information for the usage and analyses of the dengue vaccines, for which documentation of prior infection is important prior to administration, due to concerns about antibody-dependent enhancement (ADE). For example, Dengvaxia can only be used in people previously infected with DENV, and thus the competitive ELISA can identify previous DENV infections. (5) The format could be useful for vaccine development to identify the specificity to antigen variants. To this end, the UB-DNA aptamer generation targeting viral particle or envelope proteins is more helpful.

Our tests using patient samples with secondary DENV infections revealed that the IgG antibodies responding to the past infection were produced predominantly, even upon secondary infections with different dengue serotypes. These results correlate with other reports[21-24,26,27,60], supporting ADE where secondary heterologous infections occasionally result in severe symptoms and highlighting the risk of vaccinating dengue-naïve individuals. Patients with a primary infection produced IgG antibodies that mainly targeted the infected serotype. In the secondary infection, the initially produced IgG antibodies reacted more to the NS1 serotype of the past infection, and did not effectively react with the targets of the secondary infection. The application of this test in a larger cohort of dengue patients will clarify the mechanism of dengue pathogenesis, through the serotype-specific sequence of the DENV infection. In the future, our method may be expanded to test the efficacy of vaccine development[50,51], and to diagnose other diseases and allergies.

This competitive ELISA format is a simple method using UB-DNA aptamers, which enable the quantitatively identification of serotype-specific IgGs. A similar IgG detection concept using conventional

DNA aptamers was used to detect the IgG antibodies to the P48 protein of *Mycoplasma bovis*[66]. However, the affinities of the DNA aptamers to the target were relatively low (K_D = 16–33 nM), and thus the background in the IgG detection was high and the quantitative analysis was difficult.

Although the antibody–antibody sandwich system can also be used for the IgG detection, the sensitivity is lower than that of the UB-DNA aptamer–antibody sandwich system, especially in the early stage of infection (Figure 7). This sensitivity difference between these systems might result from the different binding modes between aptamer–target and antibody–target. In general, nucleic acid aptamers cover wider areas of target proteins, as compared to those covered by antibodies, and thus a series of IgGs that bind to somewhere within the wide area of the targets can inhibit the aptamer binding.

As shown in Figure 3, the commercially available human serum contains considerable amounts of anti-DEN-NS1 IgGs, presuming the widespread DENV epidemic where the samples were collected. This also draws attention to the use of serum as a control in epidemic and pandemic disease studies, such as the current COVID-19 pandemic. In our study, we used serum sample 2 (Figure 3e) as the DENV-related IgG-free control serum.

For the practical use as a serological dengue test, the further validation of our competitive ELISA format is required. Although we need further cohort follow-up studies for the determination of the sensitivity and specificity of our method, the issue is the preparation of well-characterized clinical samples without and with DENV infection, including serotypes, because of the possibility of multiple, unrealized infections. Since the first DENV infection is rarely severe, patients are often unaware of the infection or assume the symptom as a common cold. In addition, other cross-reactive viral infections, such as ZIKA, can complicate the validation, although we confirmed that our UB-DNA aptamers targeting DEN-NS1 do not bind to the NS1 proteins from several ZIKA strains[49]. Thus, the identification of DENV infection in clinical samples is not an easy task. We will validate our method by comparisons with the conventional methods and kits, using the well-characterized clinical samples with sufficient patient information.

The combination of this competitive ELISA for IgG detection with the original ELISA format for the serotype- or sub-serotype-specific DEN-NS1 detection[49] allows the diagnosis of both past and current viral infections and will facilitate early medical care. Our competitive ELISA method could be employed for a wide range of IgG detections for infectious diseases, allergenic reactions and sensitivities, and vaccine development. By generating UB-DNA aptamers targeting each antigen or its variants, the competitive ELISA format using each aptamer/target pair could identify the specificity of IgGs produced by responses to infection, allergens, and vaccine treatments. Recently, SARS-CoV-2 serological assays with competitive or blocking ELISA formats, using the interaction between ACE2 and the viral spike protein, have been utilized to detect neutralization antibodies[67,68] and are commercially available as a test kit (cPass from GenScript). However, there is a possibility that the serologic test kits have the problem of cross-reactivity between dengue and COVID-19 for both types of patients[9,10]. As noted above, UB-DNA aptamers cover a large area of target proteins, and our competitive ELISA format could detect not only the neutralization antibodies but also other IgGs that competitively bind to different target areas.

Methods

Oligonucleotides, proteins, clinical samples, and ethics statement. Biotinylated UB-DNA aptamers (AptD1, AptD2, AptD3, and AptD4, Figure 2b and Supplementary Table 1) were chemically synthesized with an H8 DNA/RNA Synthesizer (K&A Laborgerate) in-house by using natural base, Biotin-dT, and Ds and Pa phosphoramidites. The natural base and Biotin-dT phosphoramidites were purchased from Glen Research and LGC Link Technologies. The Ds and Pa phosphoramidites were prepared in-house, as described previously[52,53]. The synthesized UB-DNA aptamers were purified by denaturing gel electrophoresis after deprotection. Recombinant DENV1–4 NS1 proteins (DEN1-NS1, DEN2-NS1, DEN3-NS1, and DEN4-NS1) were purchased from The Native Antigen Company. Control human serum was purchased from Sigma-Aldrich (Sigma #H4522, pooled) and obtained from a healthy Singaporean volunteer (human serum sample 1), or two different Japanese volunteers (human serum samples 2 and 3) who have never been infected by dengue viruses.

Clinical samples (serum or plasma) used in this study were prepared from the whole-blood samples of the patients who consented to the study and provided written informed consent, referred by the Communicable Disease Center, TTSH. The study protocol was approved by the NHG DSRB (references 2015/00528 and 2016/00076). Anti-dengue NS1 rabbit monoclonal antibodies, Ab#D06 and Ab#D25, were prepared in-house[49]. The Streptavidin-HRP conjugate (1 mg/mL) and Peroxidase-conjugated AffiniPure Goat Anti-Human IgG (H+L) (#109-035-003, 1 mg/mL) were obtained from Jackson ImmunoResearch. Streptavidin, Tween 20, BSA, and anti-rabbit IgG HRP conjugate (1 mg/mL) were obtained from Promega. The 10× D-PBS solution was purchased from Nacalai Tesque.

The present experiments were approved by the institutions of A*STAR and NCID, and we confirm that all methods were performed in accordance with the guidelines and regulations of A*STAR and NCID.

ELISA using aptamer–antibody pair (Apt/Ab ELISA) for DEN-NS1 detection. All the incubation processes were performed at room temperature. Microtiter plates (Maxisorp^{TM} 96-well plates from Thermo Fisher Scientific, #442404) were coated overnight with 100 µL/well of 10 µg/mL streptavidin in 0.1 M sodium carbonate buffer (pH 9.6). The streptavidin-coated wells were blocked with 300 µL of 10 mg/mL BSA in 1× D-PBS for two hours, and then washed three times with 300 µL of washing buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 2.7 mM KCl, 0.05% Tween 20). Each UB-DNA aptamer in dilution buffer (washing buffer supplemented with 1 mg/mL BSA) was immobilized on the streptavidin-coated wells by a 2-hour incubation (100 µL, 15 nM AptD1 or 5 nM AptD2, AptD3, or AptD4), the wells were washed three times, each with 300 µL of washing buffer. To the aptamer-coated wells, 100 µL of an NS1–Ab#D06 mixture solution was added and incubated for 30 min. The mixture solutions were prepared beforehand, by a 30-min incubation of each NS1 protein in dilution buffer or human serum with 11.1 nM of Ab#D06 in dilution buffer supplemented with 2% Tween 20 (final concentration, dilution buffer 2) at 1: 9 ratios (vol/vol). After washing the wells once, 100 µL of secondary detector solution (anti-rabbit IgG HRP conjugate, diluted to 1: 2,500 with dilution buffer) was added to each well, and then incubated for 30 min. After washing the wells six times, each with 300 µL of washing buffer, 100 µL/well of SureBlue Reserve^{TM}

TMB Microwell Peroxidase Substrate (KPL) was added and incubated for 30 min. After adding 100 µL of 1 N HCl to each well to stop the reaction, the absorbance of the wells at 450 nm (OD_{450}) was measured with a microplate reader, Cytation 3 (BioTek). The assays under each condition were performed in duplicate (n = 2), and the average absorbance data are shown in the graphs with error bars, which represent one standard deviation. When at least one of the two sample wells showed overflow (OD_{450} > 4.000), the data are shown with wavy lines in the graphs.

Treatment of control human serum with protein A resin. The IgG in human serum was removed with protein A resin. Human serum from Sigma (500 µL, Lot#SLBT0310) was incubated with Amintra Protein A Resin (Expedeon, 500 µL of the slurry was washed three times, each with 1 mL of washing buffer) at room temperature for two hours with rotation. After the incubation, the resin was removed by centrifugation, and the supernatant was recovered and kept at 4°C until use.

Serology testing and DEN-NS1 detection using commercially available kits. As the controls, anti-dengue IgG and IgM serology detection and dengue NS1 detection were performed using commercially available lateral flow assays, Panbio Dengue Duo Cassette (Alere) and SD BIOLINE Dengue NS1 Ag rapid test (Alere), respectively. For high-titer IgG and IgM detection, 10 µL of each sample (human serum) was applied to the sample well of a Panbio Dengue Duo Cassette, and then two drops of the buffer included in the kit were immediately added. After 15 min, the test lines for IgG and IgM, as well as the control line, were checked visually, with the naked eye. For NS1 detection, 100 µL of each sample (human serum or human plasma) was applied to the sample well of SD BIOLINE Dengue NS1 Ag rapid test. After 20 min, the test and control lines were checked visually with the naked eye.

Competitive IgG detection using Apt/Ab and Ab/Ab ELISA formats. For the assays by the Apt/Ab ELISA format, we used the wells coated with each UB-DNA aptamer as a capture agent. For the preparation of the loading samples, a serum sample (5 µL, directly or 10, 25, 50 or 100-fold diluted with dilution buffer) was first mixed with 0.5 µL of each NS1 protein (DEN1-NS1: 350 or 200 pg, DEN2-NS1: 350 or 500 pg, DEN3-NS1: 450 or 875 pg, DEN4-NS1 200 or 275 pg). The spiked NS1 protein amounts were adjusted to each DEN-NS1 protein lot used in the assays, so that the OD_{450} values only overflowed in dilution buffer, since the detection sensitivity of the spiked NS1 varied depending on the purchased DEN-NS1's lot (Supplementary Figure 1). After a 30-min incubation at 25°C, the solution was mixed with 45 µL of 11.1 nM Ab#D06 in dilution buffer 2 and incubated for 30 min, followed by loading into the aptamer-coated well (50 µL) and a 30-min incubation. The subsequent procedures were performed as described above for the Apt/Ab ELISA.

For the assays by the Ab/Ab ELISA format, we used the wells coated with Ab#D25 (overnight) as a capture agent. For the preparation of the loading samples, a serum sample (5 µL, directly or 10, 25, 50 or 100-fold diluted with dilution buffer) was first mixed with 0.5 µL of each NS1 protein (DEN1-NS1: 400 pg, DEN2-NS1: 250 pg, DEN3-NS1: 400 pg, DEN4-NS1: 300 pg). The spiked NS1 protein amounts were adjusted by each DEN-NS1 protein so that the OD_{450} values only overflowed in the 10% control human serum sample, since the background signal levels were different between dilution buffer and 10% human

serum (Supplementary Figure 1)[49]. After a 30-min incubation at 25°C, 45 µL of the solution was mixed with 11.1 nM biotinylated Ab#D06 in dilution buffer 2. The subsequent procedures were performed as described above for the Ab/Ab ELISA.

From the plots of OD_{450} against the volume of human serum used in the ELISA, the relative IgG activity was calculated through normalization of the serum volume required to exhibit an OD_{450} lower than 1.0 (5 / [the serum volume required for an OD_{450} of 1.0]), as shown in Supplementary Figure 2.

Typical conventional method for IgG detection by ELISA. For the assays, we prepared the wells coated with each DEN-NS1, by a 2-hour incubation of 1 µg/mL DEN-NS1 (The Native Antigen Company, 50 µL/well) in 0.1 M sodium carbonate buffer (pH 9.6) at 25°C, followed by blocking with BSA. A serum sample (0.05 to 5 µL) in dilution buffer (50 µL) was loaded in the well and incubated for 30 min at 25°C. After washing the wells once, 100 µL of secondary detector solution (Peroxidase-conjugated AffiniPure Goat Anti-Human IgG (H+L), diluted 1: 2,500 with dilution buffer) was added to each well and then incubated for 30 min, followed by TMB detection as described above in the Apt/Ab and Ab/Ab ELISA formats.

Declarations

Acknowledgements

This work was supported by the Institute of Bioengineering and Nanotechnology (Biomedical Research Council, Agency for Science, Technology and Research, Singapore) and the National Research Foundation of Singapore (NRF-CRP17-2017-07).

Author Contributions

K.M. performed ExSELEX for aptamer generation. K.M., M.K., and I.H. characterized and optimized all of the UB-DNA aptamers for developing the competitive ELISA format, and prepared the manuscript and figures. W.S. generated and produced the monoclonal antibodies that recognized DEN-NS1. Y.S.L., V.W.L., and S.V. conceived the study, and collected and characterized the dengue samples. I.H. conceived and designed the study, chemically synthesized all of the UB-DNA fragments and organized and supervised the entire research program. All authors analyzed the data, interpreted the results, and read and approved the final manuscript.

Competing Interests

K.M., M.K., Y.S.L., W.S., and I.H. as the inventors filed a patent application regarding aptamers and methods that were presented in this study. The authors declare no additional financial interests.

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Figures

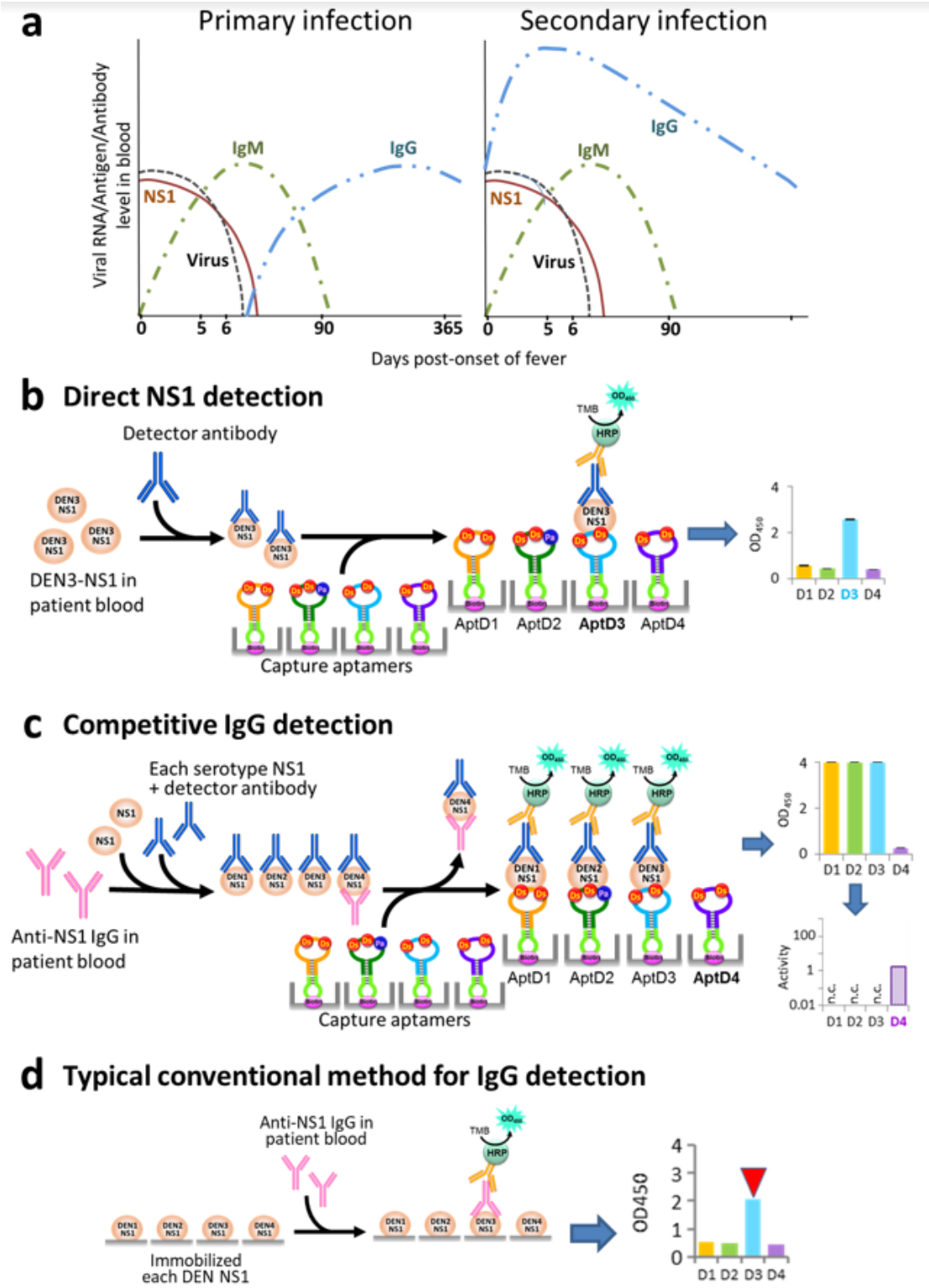


Figure 1

Dengue diagnostics using ELISA formats. (a) Schematic illustration of general detection sensitivity patterns for dengue viral (DENV) NS1 protein, virus (genome), and DENV-reactive IgM and IgG, in primary and secondary DENV infections. (b) Direct DEN-NS1 detection in patient blood, by using our UB-DNA aptamers specific to each serotype NS1 protein as capture agents and an anti-DEN-NS1 antibody (Ab#D06) as a detector agent49. (c) Competitive IgG detection in patient blood, by modifying the DEN-NS1 detection method in panel b. If a blood sample contains IgGs binding to DEN-NS1, then the DEN-NS1 detection by UB-DNA aptamers is competitively inhibited. Thus, the addition of each serotype DEN-NS1 and UB-DNA aptamer to the blood sample, followed by mixing with the detector antibody, enables the detection and quantification of IgGs specific to each DEN-NS1 serotype. (d) Typical conventional method for IgG detection, using the antigen (NS1)-coated plates to capture anti-NS1 IgGs in a blood sample.

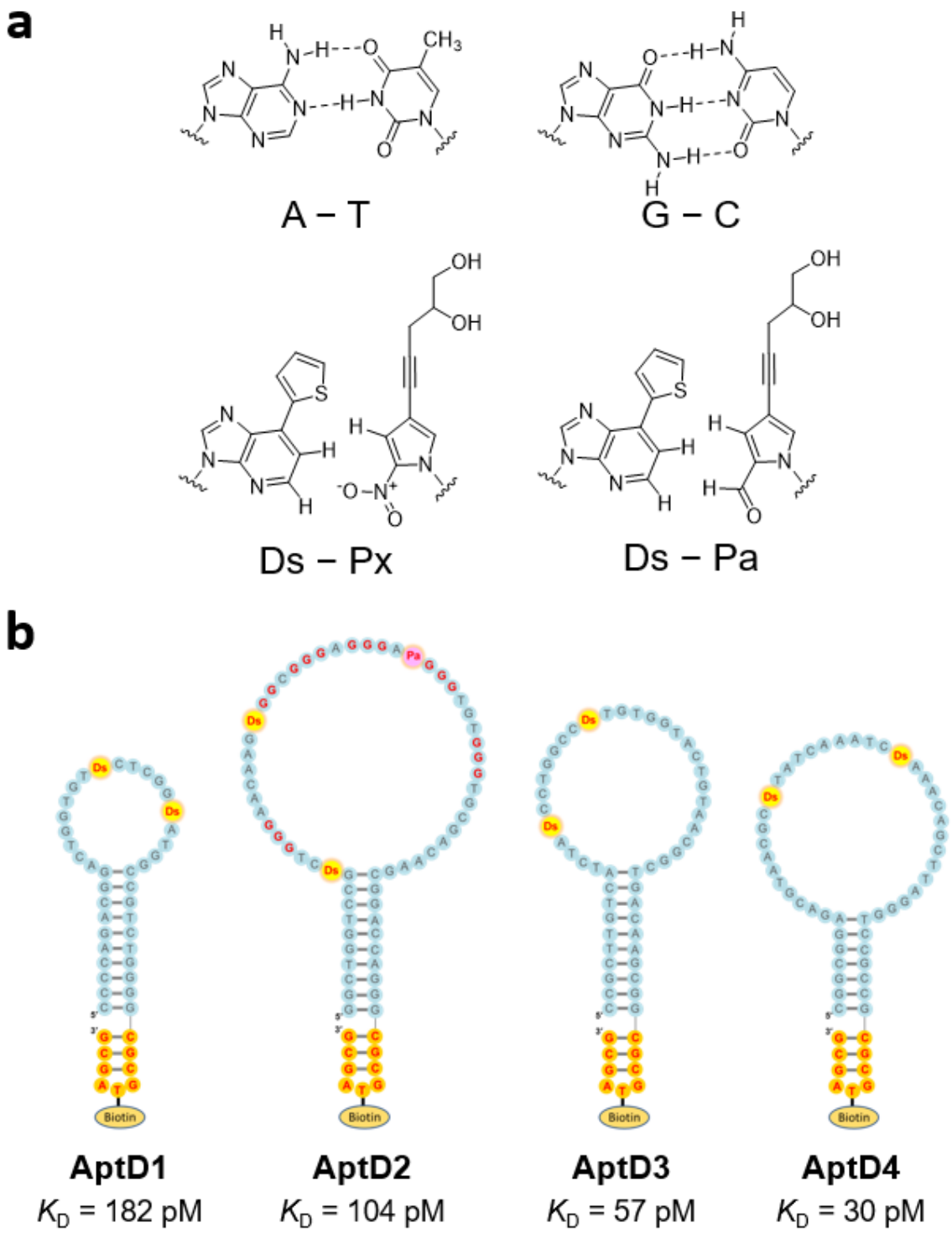


Figure 2

Natural and unnatural base pairs for UB-DNA aptamer generation and anti-DEN-NS1 UB-DNA aptamers. (a) Chemical structures of the natural base pairs, A–T and G–C, and the unnatural base pairs, Ds–Px and Ds–Pa. (b) Presumed secondary structures of UB-DNA aptamers that bind specifically to each serotype DEN-NS1: AptD1 to DEN1-NS1, AptD2 to DEN2-NS1, AptD3 to DEN3-NS1, and AptD4 to DEN4-NS1. Each aptamer's dissociation constant (KD) determined by SPR analysis is shown. The unnatural Ds and Pa

bases are shown in yellow and pink circles. At each aptamer's 3'-terminus, the stable mini-hairpin sequence (CGCG(Bio-T)AGCG) is attached for stabilization against heat and nucleases and to introduce the biotinylated T (Bio-T) for aptamer immobilization.

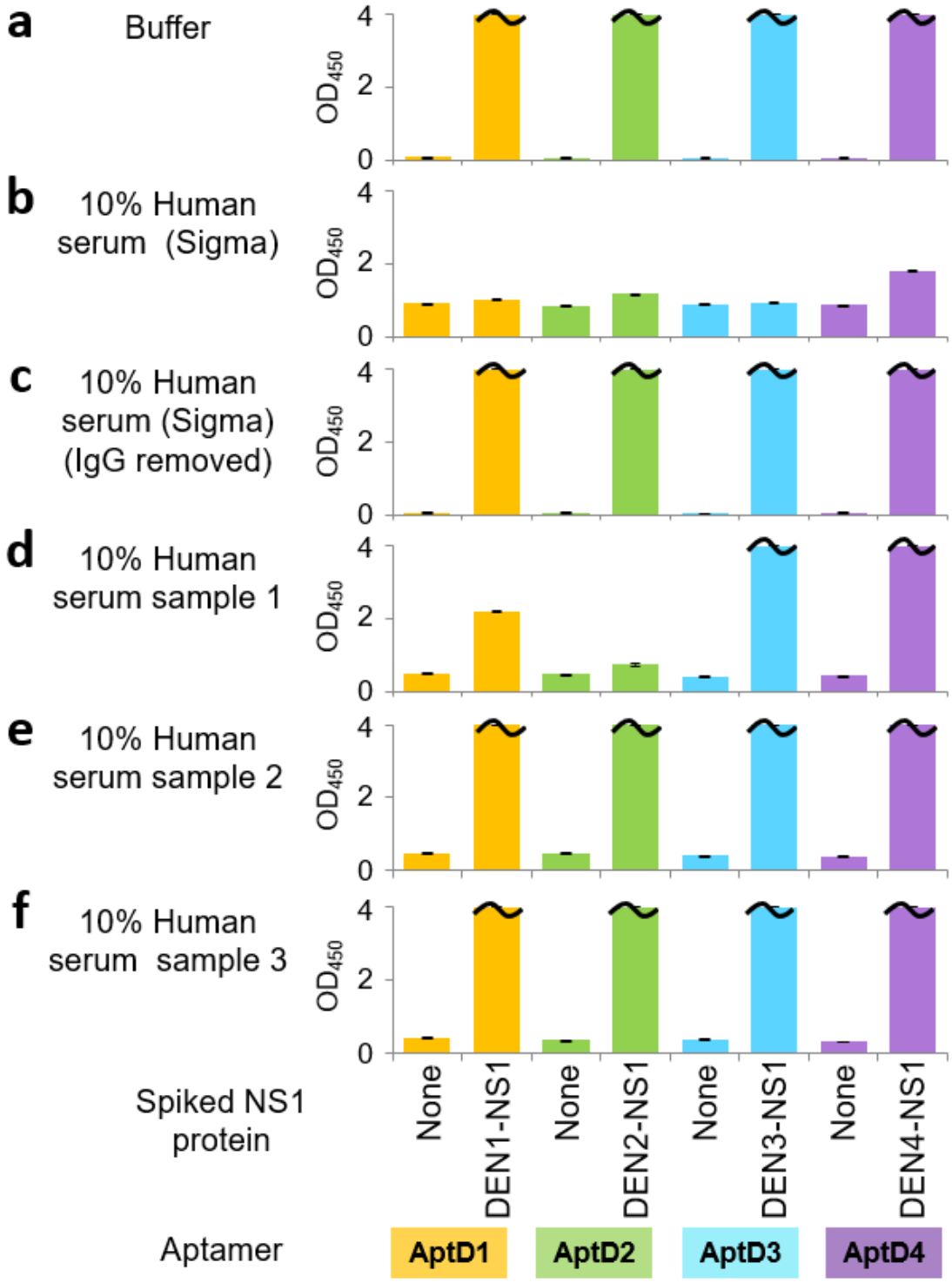


Figure 3

Inhibitory effects of human serum against direct NS1 detection. Inhibitory effects of different human serum samples were analyzed using the aptamer/antibody (Apt/Ab) ELISA format. Each UB-DNA

aptamer was employed as the capture agent, and the antibody, Ab#D06, was used as the primary detector agent. Each serotype DEN-NS1 was added in buffer (panel a) or human serum purchased from Sigma (untreated in panel b or treated with protein A resin for removal of IgGs in panel c), or each human serum sample obtained from three different persons with no current dengue infection (panels d–f). The mixtures were subjected to ELISA at the final 10% human serum concentration. A certain amount of the recombinant DEN-NS1 purchased from The Native Antigen Company (350 pg for DEN1-NS1, 350 pg for DEN2-NS1, 450 pg for DEN3-NS1, and 200 pg for DEN4-NS1) was added in each well (50 µL).

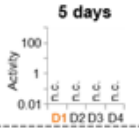
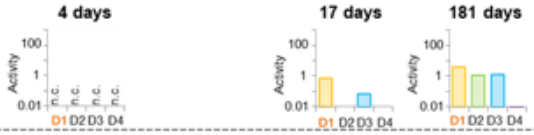
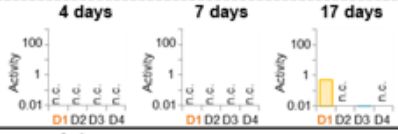
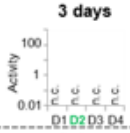
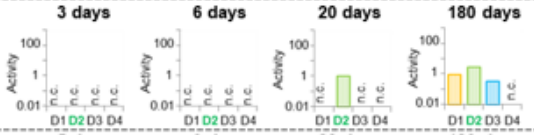
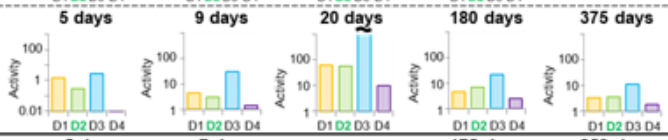
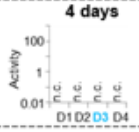
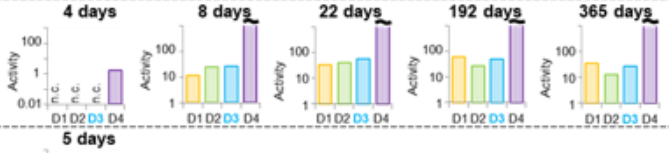
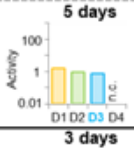
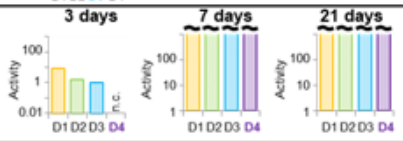
Sample ID **Current Infection** **Past infection**	**Fever day** NS1 (SD) IgM (Panbio) IgG (Panbio)	**Anti-NS1 IgG detection** (Relative inhibitory activity)
PD1-1 **Primary: D1**	**5 days** NS1: + IgM: + IgG: +	
PD1-2 **Primary: D1**	**4 days** NS1: + IgM: - IgG: -	
PD1-3 **Primary: D1**	**4 days** NS1: + IgM: - IgG: -	
PD2-1 **Primary: D2**	**3 days** NS1: + IgM: + IgG: -	
PD2-2 **Primary: D2**	**3 days** NS1: + IgM: - IgG: -	
PD2-3 **Secondary: D2** **Past: D3**	**5 days** NS1: + IgM: + IgG: +	
PD3-1 **Primary: D3**	**5 days** NS1: + IgM: + IgG: -	
PD3-2 **Primary: D3**	**4 days** NS1: + IgM: + IgG: +	
PD3-3 **Secondary: D3** **Past: D4**	**4 days** NS1: + IgM: - IgG: -	
PD3-4 **Secondary:D3** **Past: D1-3**	**5 days** NS1: + IgM: - IgG: +	
PD4-1 **Secondary: D4** **Past: D1-3**	**3 days** NS1: + IgM: - IgG: +	

Figure 4

Anti-DEN-NS1 IgG detection in Singaporean clinical samples using the competitive Apt/Ab ELISA format. The clinical human serum or plasma samples (PD1-1 to PD4-1) were obtained in 2016 in Singapore. The direct DEN-NS1 detection of the samples at the early stage (3–5 days after fever onset) was performed by the SD BIOLINE Dengue NS1 Ag rapid test. The IgM and IgG detections were performed with a Panbio Dengue Duo Cassette. The quantitative IgG activities to each serotype DEN-NS1 in the clinical samples were determined by our competitive Apt/Ab ELISA format, which also identified the current infection status (Primary or Secondary) and the serotype of the past infection (Past). The discrepancies with the IgG detection between the Panbio LFA and our competitive ELISA data are indicated in red. The RT-PCR analysis of the samples at the early stage also confirmed the DENV serotype caused by the current infection.

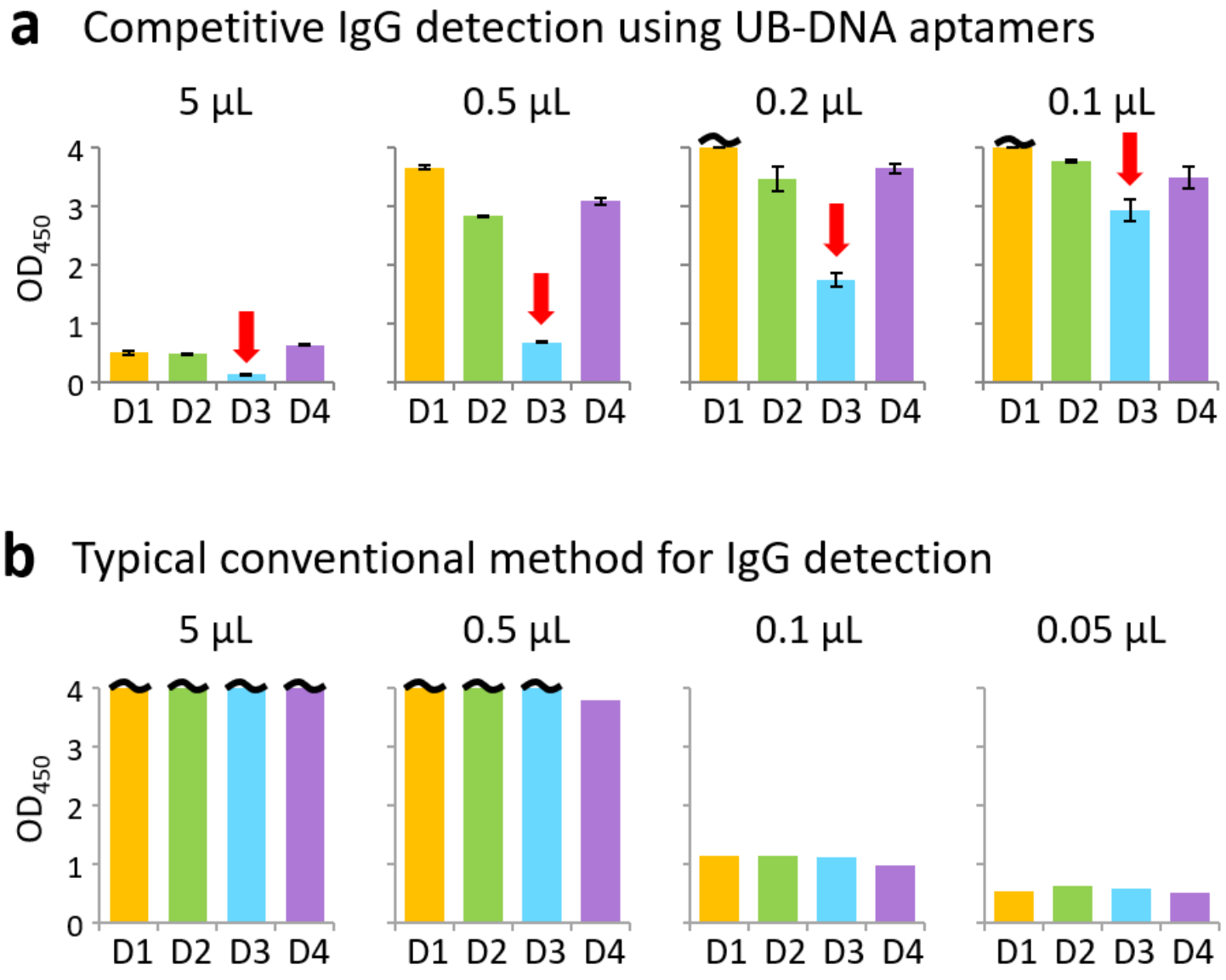


Figure 5

Comparison of the serotype-specific IgG detection by the competitive and conventional ELISA formats using a clinical sample (plasma, PD2-4). (a) The competitive Apt/Ab ELISA format (Figure 1c), displaying the inhibition of the aptamer–DEN-NS1 binding by IgGs in our competitive Apt/Ab ELISA format using 0.1 to 5 μL of a clinical sample obtained within 4 days after fever onset. The amounts of the recombinant DEN-NS1 (The Native Antigen Company) added to each well (50 μL) were 350 pg for DEN1-NS1, 350 pg for DEN2-NS1, 450 pg for DEN3-NS1, and 200 pg for DEN4-NS1. Red arrows indicate the higher inhibition of the spiked DEN-NS1 detection among the four serotypes of DEN-NS1, suggesting that the IgG in the sample is more specific to DEN3-NS1. The results revealed that the current infection of PD2-4 might be secondary, and the past infection might be DENV serotype 3, although the Panbio LFA kit did not detect IgGs from the sample (Supplementary Table 2). (b) Direct IgG detection by a typical conventional ELISA method (Figure 1d) using 0.05–5 μL of the sample. Each well was coated with 50 ng recombinant DEN-NS1 protein.

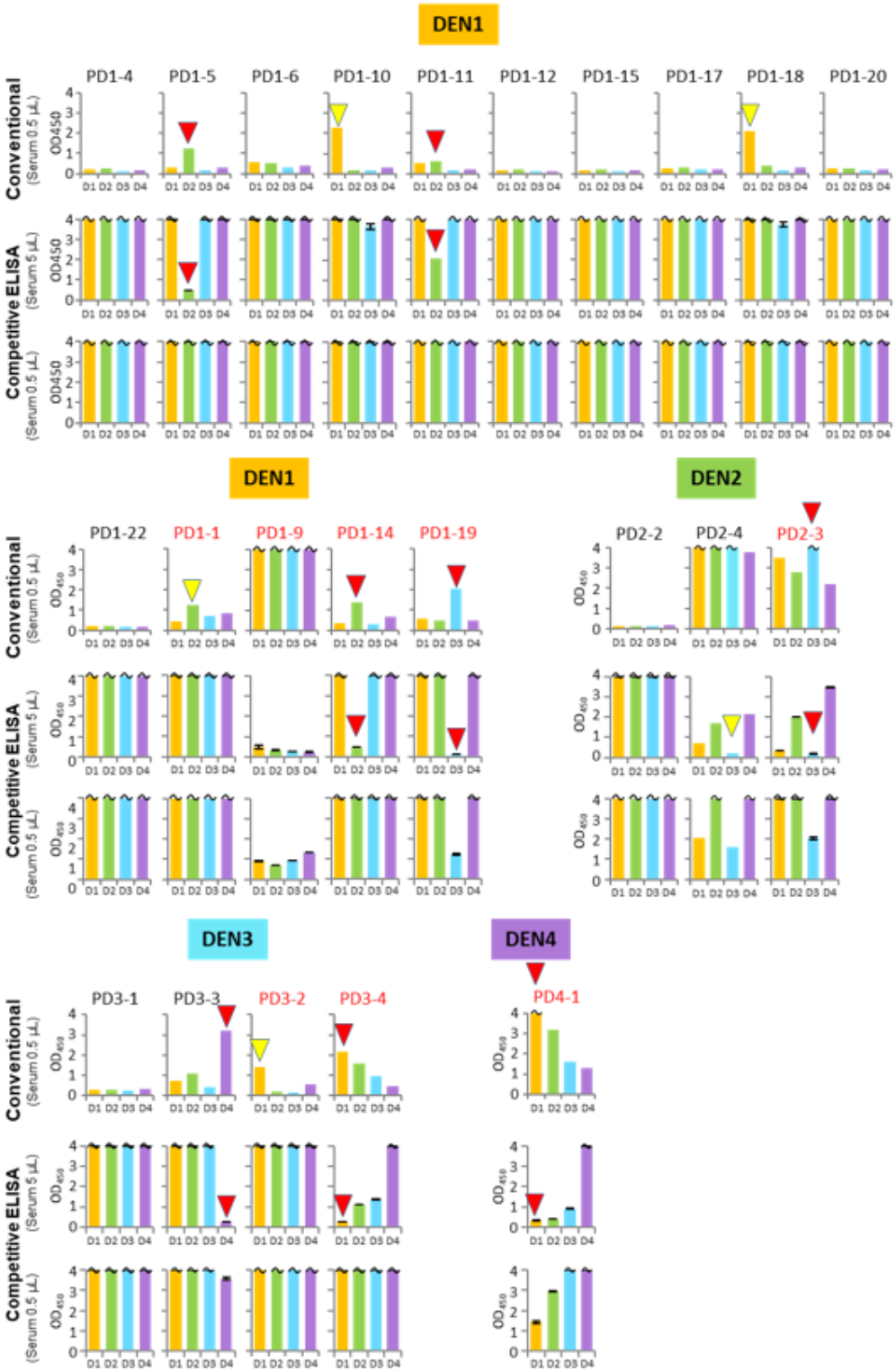


Figure 6

Comparison of the IgG detection sensitivity and selectivity using various clinical samples with two types of ELISA. Conventional: the direct IgG detection by the typical ELISA method in Figs. 1d and 5b using 0.5 μL of the serum or plasma samples. Competitive ELISA: the aptamer–DEN-NS1 binding inhibition by our competitive Apt/Ab ELISA (Figure 1c and 5a) using 0.5 μL and 5 μL of the serum or plasma samples. The amounts of the spiked DEN-NS1 are indicated in Supplementary Figure 1. The similar patterns of the

serotype-specific IgG detection by both ELISA formats are indicated by red triangles. IgG detection patterns that differ between the conventional and competitive ELISA formats are indicated by yellow triangles. The patient sample names (indicated in red) were assigned as secondary infection by the LFA test.

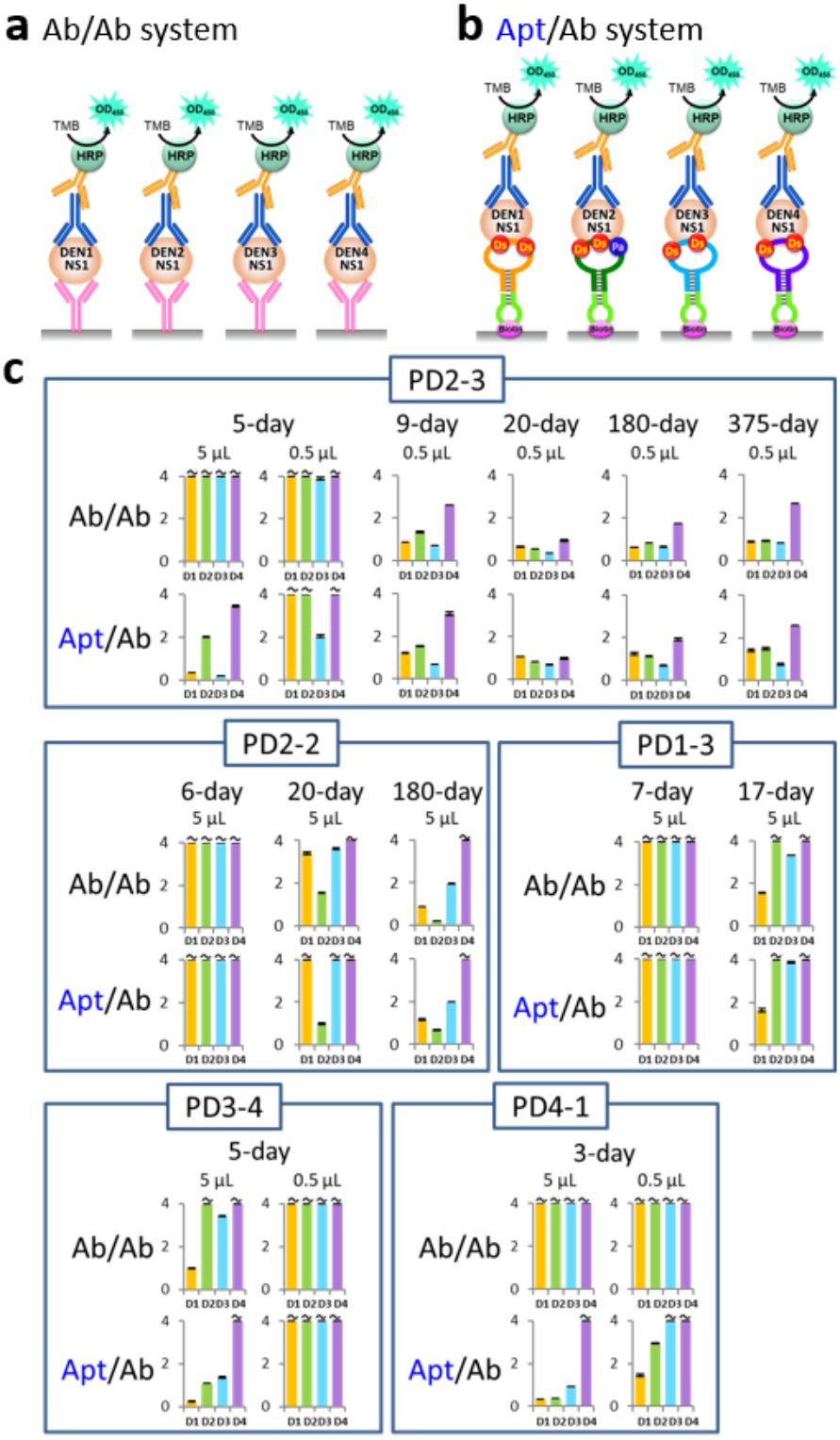


Figure 7

Comparison of the competitive ELISA formats between aptamer–antibody (Apt/Ab) and antibody–antibody (Ab/Ab) sandwich systems. (a and b) Schematic representation of the components in each system, using four DEN-NS1 serotypes. In the Ab/Ab system, the same antibodies for the capture and detection reagents were used. In the Apt/Ab system, each UB-DNA aptamer was used for each DEN-NS1 serotype. (c) Comparison of ELISA signal patterns (inhibition) of each clinical sample in different time courses.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryInformationMatsunagaetal.pdf