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Selection of DNA aptamers that bind to influenza A viruses with high affinity and broad subtype specificity

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ABSTRACT

Many cases of influenza are reported worldwide every year. The influenza virus often acquires new antigenicity, which is known as antigenic shift; this results in the emergence of new virus strains, for which preexisting immunity is not found in the population resulting in influenza pandemics. In the event a new strain emerges, diagnostic tools must be developed rapidly to detect the novel influenza strain. The generation of high affinity antibodies is costly and takes time; therefore, an alternative detection system, aptamer detection, provides a viable alternative to antibodies as a diagnostic tool. In this study, we developed DNA aptamers that bind to HA1 proteins of multiple influenza A virus subtypes by the SELEX procedure. To evaluate the binding properties of these aptamers using colorimetric methods, we developed a novel aptamer-based sandwich detection method employing our newly identified aptamers. This novel sandwich enzyme-linked aptamer assay successfully detected the H5N1, H1N1, and H3N2 subtypes of influenza A virus with almost equal sensitivities. These findings suggest that our aptamers are attractive candidates for use as simple and sensitive diagnostic tools that need sandwich system for detecting the influenza A virus with broad subtype specificities.

1. Introduction

Aptamers are artificial DNA or RNA segments that are designed to bind a specific target [1,2]. Because of various advantages over antibodies (e.g., capable of improving sensitivities by exponential amplification, lower production costs, relative ease of modification, tailored binding affinity, and reversible denaturation), aptamers are now thought to represent alternative options to antibodies for diagnostics development [3–5]. In addition to the advantages described above, aptamers are also possible to be linked with peroxidase-mimicking DNAzyme sequences [6].

Hemagglutinin (HA), which exists as trimeric spikes on the viral membrane, plays a key role in the infection process of influenza virus and is the major target for generating a of protective antibody response [7,8]. The HA acquires antigenic variation, referred to as antigenic shift or antigenic drift, resulting in a modified HA and/or neuraminidase (NA) on the influenza viruses. This mechanism interferes with effective immune responses, making it difficult to develop anti-influenza agents or diagnostic kits.

Various research groups have developed influenza aptamers [9–12]. A22, developed by Joen et al., and A10, developed by Cheng et al., can both block the binding of the virus to MDCK cells and prevent viral infection [9,11]. The protective effect of A22 is compatible with infection by other types of human influenza viral strains; however, the efficacy was lower than that in the H3-type influenza strain. Recently, subtype-specific RNA aptamers against human influenza virus have been reported [10]. These aptamers can distinguish between closely related H3N2 strains and may be useful in the genotyping of H3N2 infleunza viruses. Either way, these aptamers described above showed relatively strict specificity for the influenza virus. The binding ability of the aptamer to the targets was found to be dependent on the tertiary structure of the target [5].

In the current study, we developed DNA aptamers to detect various subtypes of influenza A viruses with high affinity. After 10 rounds of SELEX procedures, aptamers that bound to recombinant hemagglutinin (rHA) of H5N1, H1N1 and H3N2 of the influenza A virus were isolated. Among them, three aptamers (RHA0006, RHA0385, and RHA1635) were selected based on their binding

Abbreviations: AIV, avian influenza virus; ELAA, enzyme-linked aptamer assay; HA, hemagglutinin; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; NA, neuraminidase; SELEX, systematic evolution of ligands by exponential enrichment; SPR, surface plasmon resonance.

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capacities estimated by SPR assays. Furthermore, to estimate their binding properties against the influenza A virus, we developed a sandwich enzyme linked aptamer assay (ELAA) involving RHA0006 and RHA0385. This novel sandwich detection system successfully detected the various subtypes of influenza A viral particles that were antigenically distinct. These findings suggest that our novel aptamers are attractive candidates for use as rapid and cost-effective diagnostic tools with broad subtype specificities.

2. Materials and methods

2.1. Target protein and virus

His-tagged recombinant HA proteins that are expressed in HEK293 cells were purchased from Sino Biological Inc. (Beijing, PR China). His-tagged macrophage migration inhibitory factor (His-MIF) was kindly provided by Dr. S. Tsuji (Kanagawa Research Institute, Kanagawa, Japan) [12].

All experiments involving viruses were conducted at the IIT Research Institute in accordance with institutional guidelines. Each viral strain was grown in confluent flasks of MDCK cells. A/Brisbane/59/07, A/California/04/09, and A/Singapore/16/86 stocks were prepared at a multiplicity of infection (MOI) of 0.01 plaque-forming unit (PFU)/cell. A/Brisbane/10/07, A/Wisconsin/67/05, A/Moscow/10/99, A/Georgia/20/06, and Sindbis stocks were prepared at an MOI of 0.05 PFU/cell. Virus stocks were prepared in infection media ($1 \times$ MEM + 3% $NaHCO_3$, $1 \times$ MEM vitamin solution, $1 \times$ L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin). After 1 h of infection, an overlay of infection media supplemented with 2 μg/mL TPCK-treated trypsin (Affymetrix/USB, Santa Clara, CA, USA) was added to each flask. When the cytopathic effect reached approximately 100%, the supernatant was concentrated using Amicon Ultra centrifugal filter devices with a 100,000 molecular weight cut-off (MWCO; Millipore, Billerica, MA, USA) by centrifuging at 3000*g* at 4 °C for 30 min. rg A/Japanese White Eye/Hong Kong/1038/06 was grown in specific pathogen-free fertile eggs and harvested after 48 h; the virus was diluted in $1 \times$ phosphate-buffered saline (PBS), pH 7.4, with 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin. A titer was not available for this strain; hence, 1:1000 and 1:100 dilutions of the stock were used to infect the eggs. The semipurified virus was UV-inactivated, and the protein concentration was determined using the bicinchoninic assay (BCA) (Pierce Biotechnology, Rockland, IL, USA).

2.2. SELEX

DNA aptamers were selected using a previously described method with some modifications [13]. The typical procedure has been described below. TALON Superflow Metal Affinity Resin (20 μL; Takara Bio, Inc., Shiga, Japan) was mixed with 25 μg of HA proteins (A/Anhui/1/05) in 200 μL of selection buffer (SB; 50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0.01% Tween 20). The mixture was incubated for 30 min at room temperature and then washed two times with SB. The initial ssDNA pools (2000 pmol) were denatured at 95 °C for 5 min, immediately cooled on ice for 5 min, and then pre-absorbed with TALON resins in SB supplemented with tRNA and acetylated bovine serum albumin (BSA; Wako Pure Chemical Industries, Osaka, Japan). Unbound ssDNA pools separated using an ultra-free MC column (Millipore) were mixed with 100 μL of HA-coated beads, incubated for 30 min at room temperature, and then washed three times with 750 μL of SB. Bound aptamer-HA1 complexes were eluted with buffer containing 200 mM imidazole, separated using an MC column, and collected by ethanol precipitation. The collected ssDNA samples were amplified by PCR (*Ex Taq* DNA Polymerase, Takara) with biotin-conjugated reverse primers in a total volume of 400 μL. Amplified dsDNA was purified using NucleoSpin Extract II (Takara), incubated with MyOne-SA beads (Invitrogen, Carlsbad, CA, USA) in hybridization buffer (HB: 5 mM Tris–HCl, pH 7.4, 1 mM EDTA, 2 M NaCl, 0.1% Tween 20), and then washed two times in HB. For ssDNA preparation, bead-bound dsDNA was eluted with 0.1 M NaOH, neutralized with 0.1 M HCl containing 0.1 M Tris–HCl, pH 7.4, and then purified using NucleoSpin Extract II. We increased the selection pressure by lowering the number of target-immobilized beads while increasing the number of wash cycles. After 10 rounds of selection, non-biotinylated PCR products were purified and ligated to pGEM-T easy vectors (Promega, Madison, WI, USA). All DNA sequencing samples were prepared using the illustra TempliPhi DNA sequencing template amplification kits (GE Healthcare, Piscataway, NJ, USA) and analyzed using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Surface plasmon resonance (SPR) assay

All SPR analyses were performed using the ProteON XPR360 instrument (Bio-Rad, Hercules, CA, USA) at 25 °C. Each aptamer clone was attached to a poly(A_{24}) tail at the 3′ end for immobilization onto the NLC sensor chip via a 5′-biotinylated oligo (dT_{20}). HA proteins were injected as an analyte with SB. The dissociation constant was calculated according to the manufacturer's instructions.

2.4. Enzyme-linked aptamer assay (ELAA)

2.4.1. Direct ELAA

The recombinant HA proteins or semi-purified influenza viruses described above were diluted in carbonate buffer for immobilization onto wells (Maxisorp F96, Thermo Scientific/Nunc, Waltham, MA, USA), with viral protein concentrations ranging from 100 ng to 100 μg. Wells were coated overnight at 4 °C, washed once with SB, and then blocked with Protein-Free (TBS) blocking buffer (Thermo Scientific/Pierce Biotechnology) for 1 h at room temperature. The 3′-biotinylated aptamers were diluted in SB to a final concentration at 1 μM and then denatured at 95 °C for 5 min. The aptamers were added to the virus-coated wells and incubated for 1 h at room temperature. After three washes with SB, streptavidin-horseradish peroxidase (SA-HRP) (GE Healthcare) was added, and the mixture was incubated at room temperature for 30 min to detect the biotinylated aptamers captured by the immobilized HA proteins or virus. After three washes with SB, TMB solution (Moss Inc., Pasadena, MD, USA) was added for detection, and the solution was incubated for 10 min at room temperature. The reaction was stopped by adding 0.5 N sulfuric acid, and the absorbance at 450 nm of each well was measured.

2.4.2. Sandwich ELAA

Amino groups were conjugated through a C12 linker at the 3′ terminus of each aptamer; these modified aptamers were used for capture. These aptamers were diluted in carbonate buffer to a final concentration of 0.2 μM and added to each well of an amino-immobilizer plate (Thermo Scientific/Nunc). The plates were coated overnight, washed once, and then blocked with 1 M Tris–HCl, pH 7.4, for 30 min, followed by incubation in Protein-Free (TBS) blocking buffer for 1 h at room temperature. The wells were washed three times and HA proteins or semi-purified influenza viruses, diluted in SB, were added to the wells and incubated for 2 h at room temperature. The remaining procedures were the same as those for described above for direct ELAA.

3. Results and discussion

3.1. Evaluation and characterization of DNA aptamers by using the SPR assay

Primers designed for SELEX were optimized in terms of stability and multiplicity for the DNA library by using the ValFold program [14]. A nucleotide library was obtained from a pool of 10^{15} single-stranded DNA (ssDNA) molecules containing a random 30-mer sequence flanked by these designed primers. This random library was screened with recombinant hemagglutinin (rHA) proteins of the H5N1 influenza virus by using the general SELEX procedure with slight modifications (see Section 2). Among the 215 clones sequenced from the 10 round pools, 15 sequences that were overlapping by more than 5% were observed (data not shown).

To evaluate the binding affinities of these overlapping sequences, which were considered to be aptamer candidates, SPR assays were conducted. Among aptamer candidates, three clones, RHA0006, RHA0385, and RHA1635, were selected based on the binding strength to rHA. These three RHA aptamers were optimized based on secondary structures calculated using the ValFold program. The sequences of three optimized aptamers are shown in Fig. 1, and these aptamers were used in further assays. Prediction of secondary structure revealed that RHA0006 and RHA0385 formed a typical G-quadruplex structure, while RHA1635 did not form this structure (Fig. 1B) [14]. Additionally, potassium ions were required for efficient binding of both RHA0006 and RHA0385 to rHA, while RHA1635 did not require potassium ions for target binding (Supplemental Fig. 1A). These results suggest that the estimated secondary structures are reliable.

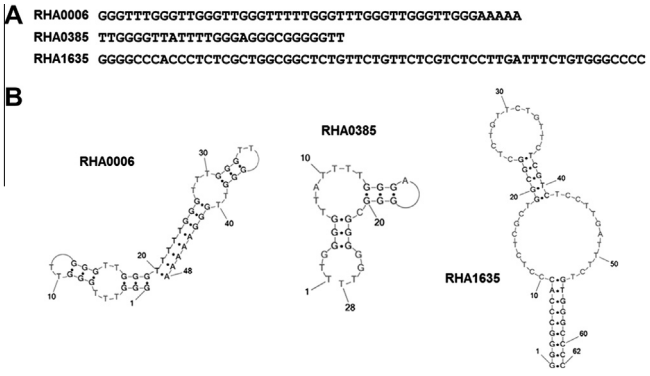

Fig. 1. Sequence (A) and secondary structure calculated by the Valfold program (B). ValFold predicts not only canonical Watson–Crick pairs but also G–G pairs derived from the G-quadruplex by using the stem candidate selection algorithm.

As shown in Fig. 2A, the three aptamers bound to not only the extracellular domain of rHA (HA1 + 2) but also the subunit 1 of rHA (HA1) of the same H5N1 virus (A/Anhui/1/2005). The signal intensity of HA1 + 2 was nearly twice that of HA1. The dissociation constant (K_D) of RHA0006 was calculated to be 1.53×10^{-8} M (rHA1, A/Anhui/1/2005) and 2.47×10^{-8} M (rHA1 + 2, A/Anhui/1/2005), respectively. Since these K_D values were similar, the difference in signal intensity may reflect the molecular weight of HA1 + 2 (approximately 70 kD) and HA1 (approximately 40 kD). The binding site of the aptamer was thought to a region of HA1.

Next, to evaluate the binding specificity of the aptamer, rHAs of various subtypes of influenza A viruses were used as analytes. As shown in Fig. 2B, our three aptamers strongly bound to rHA of not only AIV but also of the seasonal strain (H1N1, H3N2) and pandemic strain (H1N1pdm). The three aptamers showed a similar rank order of binding affinity for each rHA1. Since they did not recognize His-MIF, which was used as a negative control, the aptamers did recognize not the His-tag but were specific to the HA1 region. Although amino acid homologies of HA1 domain between AIV and other subtypes are 36–52%, the HA1 domain contains many conserved region among the strains examined. Thus, broad specificity of RHA aptamers is feasible. A22 has previously been reported to be capable of binding to HA1 of the H3 and H2 subtypes of the influenza A virus [11]. A10 has also been reported to bind to HA1 of H5N1, which is the strain used in our binding assay [9]. Under our experimental conditions, both A10 and A22 reacted with these HA proteins compared to negative control aptamers, but both binding affinities were significantly lower than those of our RHA aptamers.

3.2. Evaluation and characterization of DNA aptamers by ELAA

To evaluate the binding properties of the aptamers using colorimetric methods, we conducted an ELAA. First, rHA proteins were

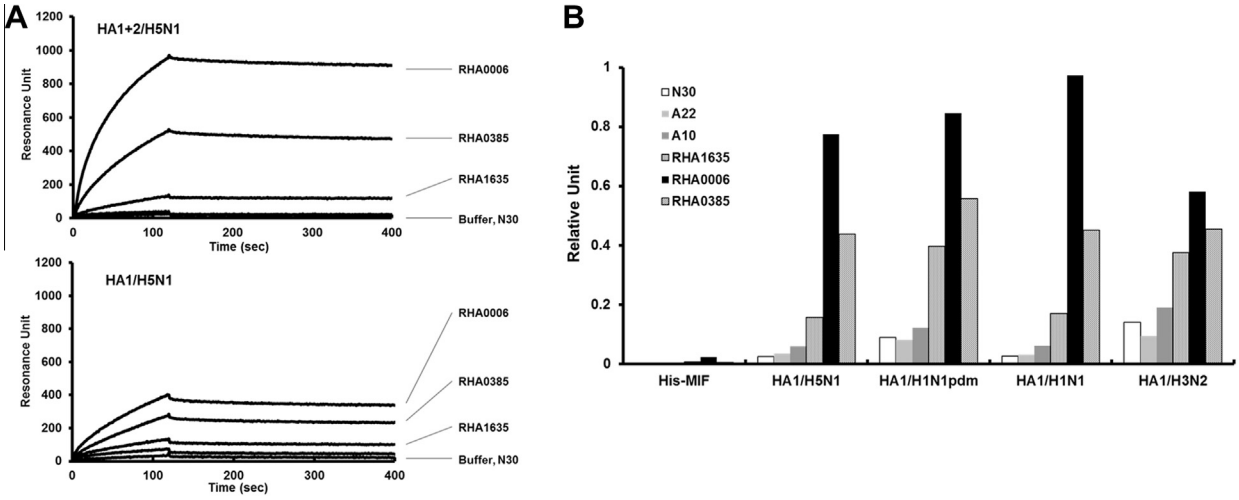

Fig. 2. Binding capacity of RHA aptamers toward rHA was measured using a SPR assay. (A) SPR assay was performed with 3′-poly(A_{24})-tailed aptamers immobilized onto a poly(dT_{20})-coated streptavidin sensor chip. RHA0006, RHA0385, and RHA1635 were our newly identified HA aptamers, whereas N30 indicates the initial unselected random oligonucleotide used in the SELEX procedure. First, 500 nM of the HA1 subunit of H5N1(A/Anhui/1/2005) or the full-length extracellular domain of HA proteins of H5N1 (HA1 + 2, A/Anhui/1/2005) in selection buffer were injected at a flow rate of 50 μL/min. (B) Here, 500 nM of HA1 subunit of H5, H1, and H3 subtypes of influenza A virus (A/Anhui/1/2005, A/California/04/2009, A/Brisbane/59/2007, A/Aichi/2/1968) in selection buffer were injected as analytes. A22 has been reported by Joen et al., and A10 has been reported by Cheng et al. Relative units were calculated by resonance units at the end point (120 s) of analyte injection, normalized to the amount of immobilized aptamer.

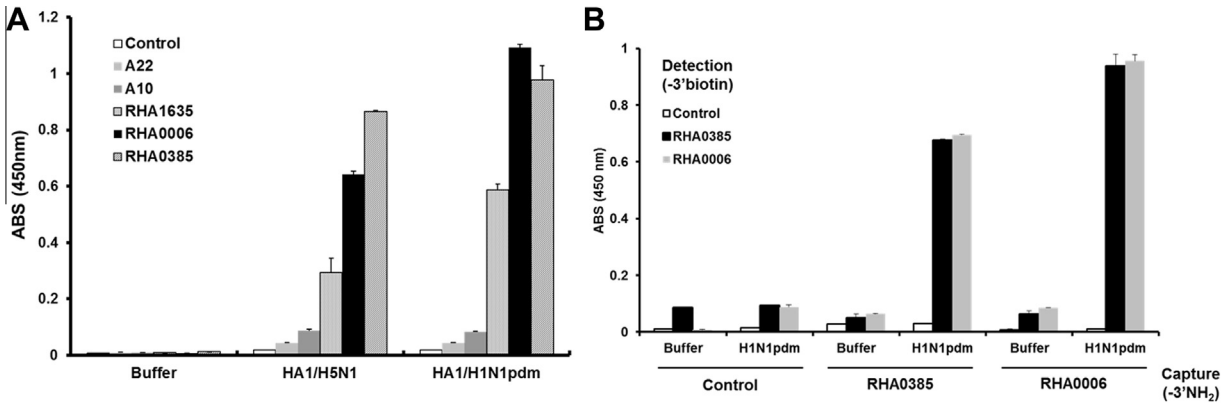

Fig. 3. Binding capacities of RHA aptamers as well as of control ssDNA toward the HA1 subunit of the H5N1 subtype (A/Anhui/1/2005) and H1N1 pandemic strain (A/California/04/2009) were evaluated by ELAA assays. (A) Direct ELAA. The amount of biotinylated aptamer captured by immobilized HA1 proteins was determined from the absorbance at 450 nm. (B) Development of effective sandwich-ELAA requiring only our RHA aptamer sequences for detecting the HA1 subunit. Here, 100 nM of 3′-aminated aptamers (–3′NH_2) was immobilized to the wells and used for capture. After two sequential blocking processes, 1 μg of recombinant HA1 proteins (A/California/04/2009) in selection buffer was added to the aptamer-coated wells, and then 1 μM of 3′-biotinylated aptamers (–3′biotin) was reacted. The amount of biotinylated aptamer bound to HA1 proteins captured by aminated aptamers was determined from the absorbance at 450 nm. The experiment was repeated three times with each data point measured in duplicate; representative data are shown.

immobilized onto a plate and then reacted with biotinylated aptamers. As shown in Fig. 3A, our aptamers showed significant binding to rHA1 of both H5 and H1 pandemic strains compared with the control aptamer. Our aptamers showed greater affinity than both A10 and A22. The detection limit of HA proteins in ELAA using rHA aptamers was 0.1 μg/well (data not shown). These results suggested that our three aptamers can be used to detect plate-immobilized rHA with high affinity.

Several sandwich systems for thrombin detection employing aptamers have been recently reported [15,16]. We confirmed that our newly identified aptamers were capable of binding to rHA1 with broad specificities, not only in solution (Fig. 3A) but also in the solid phase (Fig. 2); therefore, we attempted to develop a novel sandwich ELAA for hemagglutinin detection. Amino groups were attached through a C12 linker at the 3′ terminus of each aptamer and were used as capture. rHA1 captured by plate-immobilized aptamer was detected using a biotinylated-detection aptamer. As shown in Fig. 3B, the sandwich procedure, which involved RHA0006 and RHA0385, successfully detected rHA1 with low background signal. Because aptamers are evolved in solution, any modification altering the chemical identity of the aptamer can affect its folding and consequently its target binding. Fortunately, both RHA0006 and RHA0385 effectively functioned as both capture aptamers and detection aptamers, although the capturing capacity of RHA0385 was slightly lower than that of RHA0006. This novel sandwich-ELAA also detected rHA1 of the H5 strain, while RHA1635, which did not form the typical G-quadruplex structure, was difficult to capture and detect, possibly due to modifications or a lower sensitivity compared with other RHA aptamers (data not shown). Surprisingly, regarding both RHA0006 and RHA0385, only a single aptamer sequence was sufficient for developing sandwich detection. In this regard, we have speculated that rHA may have more than one binding site (e.g., carbohydrate chain) for these aptamers.

3.3. Characteristics of the binding of RHA aptamers to influenza A viruses

Because we succeeded in detecting rHAs using the ELAA, which was composed of RHA aptamers, we next evaluated the binding affinity and specificity of both RHA0006 and RHA1635 toward UV-irradiated influenza A virus by ELAA. First, direct ELAA was performed. Serial dilutions of the semipurified influenza virus were directly immobilized onto each well of a 96-well plate with carbonate buffer. Because AIV (A/Japanese White Eye/Hong Kong/1038/06) was grown in specific pathogen-free fertile eggs and

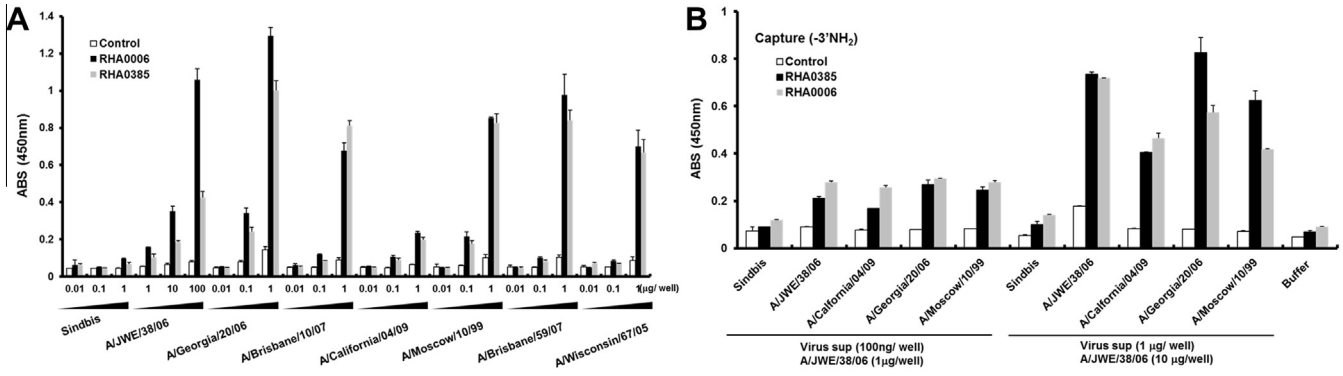

Fig. 4. Binding capacities of RHA0006 and RHA0385 toward various subtypes of influenza A virus were measured by ELAA. (A) Ten-fold serial dilutions of semipurified virus supernatant (1, 10, and 100 μg/well of H5N1 virus, and 0.01, 0.1, and 1 μg/well of other viruses) were immobilized on 96-well plates with carbonate buffer. The Sindbis virus was used as the non-influenza A virus control. (B) Sandwich-ELAA effectively detected the various subtypes of influenza A virus. Here, 100 nM of 3′-aminated aptamers (–3′NH_2) was immobilized onto the wells and used for capture. Different concentrations of semipurified virus supernatant (1 and 10 μg/well of H5N1 virus and 0.1 and 1 μg/well of other viruses) in selection buffer were added to the aptamer-coated wells, and then 1 μM of 3′-biotinylated RHA0006 aptamers (–3′biotin) were reacted. The remaining procedure for ELAA was the same as that described in Fig. 3. The experiment was repeated three times with each data point measured in duplicate, and representative data are shown.

the supernatant contained a high concentration of contaminants derived from fertile eggs, AIV was used at a 100-fold concentration, as determined from the preliminary estimation shown in Supplemental Fig. 1B. As shown in Fig. 4A, both RHA0006 and RHA1635 showed significant binding to all subtypes of influenza A viruses in a dose-dependent manner. A significant difference was observed between the binding capacity of the pandemic strains (A/California/04/09) and other influenza A virus strains for both RHA0006 and RHA0385. This tendency comparably improved when evaluated by sandwich ELAA (Fig. 4B), and the binding signal of RHA aptamers toward rHA1 of the same pandemic strains was equivalent to those of other seasonal strains (Fig. 2B); this is likely because physical adsorption of pandemic strains negatively affected the interaction between the aptamer and the pandemic strain, although the mechanism was unclear.

Finally, we examined whether our novel sandwich-ELAA could detect the influenza A virus. As shown in Fig. 3B, both RHA0385 and RHA0006 could detect the influenza A virus, specifically in a dose-dependent manner. Importantly, the binding affinities of both RHA aptamers toward AIV were 10-fold higher than those measured by direct ELAA, as shown in Fig. 4A. In direct ELAA, the AIV sample required 100-fold higher concentration than that of other influenza A viruses to generate an equivalent binding signal, and this tendency was reproducible. Since the AIV sample was not purified from fertile eggs, AIV itself could be immobilized more efficiently because the capture aptamer purified AIV in the presence of a high concentration of contaminants.

We succeeded in generating DNA aptamers that target HA proteins of the influenza A virus with high affinity and broad subtype specificity. Furthermore, we developed a selective, sensitive sandwich-ELAA employing only RHA aptamer sequences for detecting the influenza A virus. Infection experiments, including a hemagglutination inhibition test (data not shown), indicated that the RHA aptamers did not show antiviral activity. However, the binding affinities of RHA aptamers were remarkably higher than those of A22 or A10 (Figs. 2 and 3). Since our RHA aptamers showed significant and specific binding to broad subtypes of influenza A viruses, we will evaluate the binding properties against the H7N9 subtype, which has recently been found to cause human infections in China [17].

Because of the specificity and low detection limits, double-antibody sandwich detection is commonly used in clinical diagnostics, including chromatography or colorimetric enzymatic assays. In addition, besides thrombin detection described above, consecutive reports in 2013 regarding aptamer-based sandwich systems for whole viruses [18], antibodies [19], and small molecules [20] have suggested the feasibility of using aptamers as diagnostic tools. Although the sandwich ELAA described here will need to be further characterized to determine the limit of detection and subtype specificity as a simple and sensitive influenza A virus diagnostic tool, our novel aptamer assay will be one candidate applicable to a rapid and cost effective diagnostic, requiring broad subtype specificities.

Conflict of interest

The authors have no financial conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.041.

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