

SELEX-Based Direct Enzyme-Linked Aptamer Assay (DELAA) for Diagnosis of Toxoplasmosis by Detection of SAG1 Protein in Mice and Humans

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Abstract

Background: *Toxoplasma gondii* is a single-celled parasite commonly found in mammals. Diagnosis of toxoplasmosis largely depends on measurements of the antibody and/or antigen and *Toxoplasma*-derived DNAs due to the presence of tissue dwelling duplicating tachyzoites, or quiescent cysts in latent infection of the parasite. As a major surface antigen of *T.gondii* tachyzoites, SAG1 is a key marker for laboratory diagnosis. However, there are no methods available yet for SAG1 detection using aptamer-based technology.

Methods: Recombinant truncated SAG1 (r-SAG1) of *Toxoplasma* WH3 strain (type Chinese 1) was prokaryotically expressed and subjected to the synthetic oligonucleotide library for selection of nucleic acid aptamers which target the r-SAG1, with systematic evolution of ligands by exponential enrichment (SELEX) strategy. The specific aptamer-2 was screened out and used in direct enzyme-linked aptamer assay (DELAA) for detection of native SAG1 obtained from tachyzoite lysates (n-SAG1), mouse sera of acute infection, and human sera that had been verified to be positive for *Toxoplasma* DNAs by PCR amplification.

Results: The soluble r-SAG1 protein was obtained from *E.coli* lysates by purification and identification with immunoblotting, and then labelled with biotin. The selected aptamers were amplified by PCR, followed by DNA sequencing. The results showed that the aptamer-2, with the highest affinity to n-SAG1 in the sera of animals in the four aptamer candidates, has a high specificity and sensitivity when used in detection of n-SAG1 in the sera of humans when compared with the commercial kit of ELISA for *Toxoplasma* circulating antigen test.

Conclusions: A new direct enzyme-linked aptamer assay (DELAA), with aptamer-2 as the recognition probe, was developed for detection of native SAG1 protein of *Toxoplasma*. With increased sensitivity and specificity, stability, easy and cheap preparation, the aptamer-based technology is considered as a efficient method for the diagnosis of active and reactivated toxoplasmosis.

Background

Toxoplasma gondii is an obligatory intracellular parasite which can infect all warm-blooded animals, including humans. It is estimated that a quarter of world's population are chronically infected with *T.gondii* [1] although more than 80% of immunocompetent individuals of the infection are asymptomatic. The recrudescence of latent infection may cause severe clinical disease in the immunocompromised individuals such as those with HIV/AIDS [2], chemotherapy [3], long-term immunosuppressive treatments [4], and recipients who received organ transplants [5]. The main forms of toxoplasmosis in the patients include severe ocular, neurologic, and sometimes systemic diseases. Primary infection with *T.gondii* during pregnancy, particularly in the first trimester, may cause stillbirths, miscarriages, or fetal abnormalities [6].

Most cases of *Toxoplasma* infection elude diagnosis due to the lack of distinct clinical manifestations and the difficulty in obtaining specimens from individuals with chronic infection, and the simple methods used in pathogenic detection. Thus, the commonly used laboratory methods for routine diagnosis of toxoplasmosis include serological assays and *Toxoplasma* DNA detection by PCR amplification or recently, by DNA metagenomics sequencing [7]. A variety of serological assays, such as enzyme linked immunosorbent assay (ELISA), immunofluorescence (IF) and modified agglutination test (MAT) are frequently used in toxoplasmosis diagnosis [8]. Serological tests are usually retrospective and is used to evaluate the immune status in some situations but may not be validated for identification of current infection or curative effect evaluation. In conventional serological methods of ELISA, IF or MAT, total extracts or whole tachyzoites are used as antigens, which consist of cytoplasmatic and membrane components, recombinant peptides, or the parasites obtained from cell culture. These antigens fail to distinguish sera of patients with cerebral toxoplasmosis from asymptomatic infection of immunocompetent individuals, with undetectable tachyzoites in the blood. Thus, the treatment is mostly initiated on a presumptive diagnosis based on clinical and imaging features. Additionally, the forementioned methods possess inevitable shortcomings as biomarkers of detection in terms of antigen/antibody production, test cost and stability. Thus, there is an increasing need for a method which has high sensitivity and specificity, stability, affordability and simplicity to help diagnose congenital infection and distinguish between chronic and current infections.

An alternative diagnostic method for current *Toxoplasma* infection is to examine circulating antigens produced by the parasite [9]. These excretory/secretory antigens (ESAs), as a group of the most important molecules from *T. gondii*, are the majority of the circulating antigens in sera of acute or reactivated infection in immunocompromised individuals. Among them, surface antigen 1 (SAG1) is a stage-specific antigen of tachyzoite, with a high immune reactivity and immunogenicity. It probably represents the most explored and used antigen of *T.gondii* for development of serological tests [10] and preventive vaccine due to its abundance on the cell surface, reaching 3–5% of total proteins [11]. A real-time PCR assay using a set of primers targeting the SAG1 gene showed high sensitivity for the fast and specific detection of *T. gondii* [12]. However, little is known concerning the detection efficacy of the SAG1 protein circulating in blood for the diagnosis of toxoplasmosis.

Nucleic acid aptamer is a small single-stranded DNA (ssDNA) or RNA, identified and selected from a synthetic oligonucleotide library with a length of 20–80 bases *via* systematic evolution of ligands by exponential enrichment (SELEX) [13]. Aptamer may be termed as “chemical antibody” due to its non-protein-based alternative to antibodies and applied to many assays where antibodies are used, in light of its low cost of production, no animal ethic issue, high binding affinity and specificity, reliability, and easy chemical modification. Aptamer is even more flexible when real-time or on-site detection is needed, and has been used for diagnosis and therapeutics of a variety of diseases in recent years. For instance, aptamer, as a practical probe, has presented excellent efficacy in detection of the whole cells or toxins/proteins of bacteria, viruses, fungi, prion proteins, and protozoon parasites [14]. A promising result has been noted in aptamer-based detection of *T.gondii*. Two aptamers (TGA6 and TGA7) were employed, as capture probe and detection probe, in a quantum dots-labeled dual aptasensor (Q-DAS). The TGA6-

anti-*Tox*lgG-TGA7 sandwich complex was formed on the microplate and fluorescence could be read out using quantum dots as the fluorescence label. This dual-aptamer-based biosensing of *Toxoplasma* antibody detection showed a high sensitivity, specificity and affinity toward anti-*Toxoplasma* IgG antibodies compared with the routine antibody-based immunoassays [15]. It has been known that rhopty 18 (ROP18) is a critical virulence factor of *T.gondii* which is located on the parasitophorous vacuoles membrane (PVM) on the parasite invasion to its host cells. A previous investigation used aptamers of AP001 and AP002 targeting ROP18 protein to set up an enzyme-linked aptamer assay (ELAA) platform, indicating a high affinity and specificity to the antigen of RH strain and recombinant ROP18 in comparison with negative controls [16].

Theoretically, SAG1 can be easily detected as per its location and abundance on the surface of tachyzoites. However, there are no aptamer-based methods available for the measurement of SAG1 protein in sera of infected animals and humans. Here, we demonstrated that the Selex-derived aptamer-2 is able to target the native SAG1 of *Toxoplasma* (n-SAG1) WH3 strain of type Chinese 1, a predominant genotype prevalent in China, and can be used for the diagnosis of toxoplasmosis. We primarily utilized aptamer-2, which was screened out from the modified oligonucleotide library by recombinant truncated SAG1 (r-SAG1) protein, in the direct enzyme-linked aptamer assay (DELAA) to detect the n-SAG1 in total lysates of WH3 strain (WH3Ag), and in serum samples of mice with acute infection of virulent strain(WH3) and low virulent cyst-forming strain (WH6) at different doses and different times [17]. Our results showed that aptamer-2 could recognize the n-SAG1 protein with high specificity, sensitivity, and repeatability in measurement of either r-SAG1 or n-SAG1 when compared with BSA negative control. The newly developed aptamer-based sensing platform for SAG1 testing will deepen understanding of the interaction of the parasite and its host cells and for the early diagnosis of acute or reactivated toxoplasmosis in animals and humans.

Materials And Methods

Parasite culture and cell lysate preparation

Human foreskin fibroblasts (HFF, ATCC® SCRC-1041™) cells were cultured in DMEM medium containing 10% fetal bovine serum, 100 µg/ml streptomycin and 100 U/ml penicillin. After the cells reached 85% confluency, the virulent WH3 strain tachyzoites of *T. gondii* (type Chinese 1), which dominates in animals and human in China [18], were added to the plates and cultured in CO₂ incubator at 37°C. The parasites were purified by three times of washing and centrifugation. Cell lysates were prepared by combining freeze/thaw treatments and ultrasonication followed by centrifugation. The supernatants were collected and stored at -80 °C as WH3 strain parasite antigen (WH3Ag). The cysts of low virulent WH6 strain (type Chinese 1) were harvested from the brain tissues of mice under euthanasia after 6 weeks of cyst gavage.

Animal infection and human serum collection

The 8- to 10-week-old male BALB/c mice (SPF) were purchased from the Animal Center of Anhui Medical University (AMU) and had free access to sterilized water and food under standard conditions. The experiment protocols were conducted with approval of the Animal Ethics Committee of Anhui Medical University (Permission No. AMU26-081108). The mice were treated strictly in compliance with the Chinese National Institute of Health Guide for the Care and Use of Laboratory Animals. All of the animal experimental procedures were performed in licenced Biosafety II Laboratory. Thirty-five mice were inoculated intraperitoneally with 100 tachyzoites suspended in 100 μ L of PBS, and five mice were injected with 100 μ L PBS were taken as control. Sera were collected from the animals under euthanasia on days 1, 2, 3, 4, 5, 6 and 7 after infection. Additionally, for infection of low virulent WH6 strain of *Toxoplasma*, 90 mice were divided into three groups (n=30/group), and were infected with 10, 30, and 60 cysts for each through gavage, taken as the low, medium, and heavy dose infections, respectively. Five animals in each group were sacrificed under anesthesia on days 1, 3, 5, 7, and 14 post-infection. The sera were collected and stored at -80 °C for next use.

Fifty human serum samples were collected from the surplus sera after routine prenatal examination of pregnant women and the children with suspected congenital toxoplasmosis. The sera collection was approved by the Clinical Trial Ethics Committee of the Second Hospital of Hefei, Anhui (Approval No. 2020-Ke-054). The informed consents were obtained from all of the participants or their guardians in the study. Sera were subjected to PCR amplification for *Toxoplasma* DNAs (*Toxoplasma* PCR) and the results were taken as golden standards in comparison with *Toxoplasma* circulating antigen detected by ELISA (*Toxoplasma* CAg-ELISA) [19] and DELAA test kit.

Preparation of r-SAG1 and its biotin-labeling *in vitro*

The truncated SAG1 encoding gene (*sag1*) (forward primer: 5'- GGATCCTTCACTCTCAAGTGCCCTA- 3' and reverse primer: 5'- AAGCTTCCTGCA GCCGGAAACT-3') was amplified using *Toxoplasma* cDNA as template, ligated to the vector (pET32a), and transformed into *E.coli* BL21 host cells. The r-SAG1 expression in the host cells was induced by IPTG and the cells were sonicated at 5s intervals and centrifuged at 12 000 rpm for 20 min to obtain the supernatants containing r-SAG1 protein. The r-SAG1 protein with 6 \times His tags was purified using nickel column (Millipore, USA) from the supernatants. The r-SAG1 protein was separated by 10% separation gel in SDS-PAGE and transferred onto nitrocellulose membrane (NCM), followed by blocking with 5% of milk on a shaker for 90 min. The NCM was incubated with primary antibodies against r-SAG1 (1:1 000 dilution) at 4 °C for overnight and washed with TBS-T for three times followed by incubation with the HRP-conjugated secondary antibody, with slight shaking for 90 min. Sample stripes were visualized using enhanced chemiluminescence. The experimental data were analyzed using Image J 1.46 software.

For biotinylation of the r-SAG1 protein, 1.875 μ L 20mM EZ-Link NHS-PEG4-Biotin (Thermo, No. 21329, USA) was added to 0.1 mL 0.5mg/mL r-SAG1 protein at 4 °C for overnight, ensuring that one r-SAG1 protein could be labeled with 3-5 biotin molecules. The r-SAG1 protein was dialyzed in PBS to remove

excessive NHS-PEG4-Biotin, and NHS-PEG4-Biotin-labeled (biotinylated) r-SAG1 was used for subsequent aptamers screening.

Screening of ssDNA aptamers against r-SAG1 *in vitro*

Aptamer microsphere library (containing 10^9 microspheres and 10^4 - 10^5 repeats) was purchased from AM-Biotech, USA. The oligonucleotides of the library differ in 3-D conformation from those commonly used, due to the modifications with Indole-dU (W), Phenol-dU (Y), and Amine-dU(X) to improve the probability of aptamer harvests with high affinity.

The aptamer microsphere library was mixed with 2 mL buffer A (containing 0.5nM BSA and 1nM $MgCl_2$) and heated at 95°C for 5 min, then cooled at room temperature to create folded ssDNA. M-280 streptavidin-magnetic particles (SMps) (Thermo, No.11205D, USA) were added to the aptamer microsphere library and slightly shaken at 150 rpm at 37 °C for 30 min, in order to remove the aptamers that could bind to M-280 SMps. Next, the biotinylated r-SAG1 protein was combined with unused M-280 SMps and then added to the ssDNA library for positive screening of specific aptamers. The M-280 SMps-biotin labeled r-SAG1-aptamer complex was mixed with 50 μ L 1nM NaOH and incubated at 65 °C for 30 min. Then 40 μ L 2mM Tris-Cl was added to the complex for dissociation of specific aptamers for secondary screening. The dissociated solution was divided into 3 tubes, with 15 μ L each (**#1**: initial solution control, with additional 135 μ L buffer A ; **#2**: 100nM protein, with additional 30 μ L r-SAG1 and 100 μ L buffer A; **#3**: positive SMps control, with additional 130 μ L buffer A), and was incubated at room temperature for 1h. Five microliters of SMps were added to groups #2 and #3, respectively, and incubated at room temperature for 30min. The groups #2 and #3 were placed on magnetic racks to collect the filtered aptamers. Afterwards, the products of the three tubes (**#1**, **#2** and **#3**) were used for PCR amplification. The three groups were amplified separately by PCR containing 10 μ L 10 \times PCR buffer, 2.5 mM $MgCl_2$, 0.4 M, with same forward primer (K-FP: 5'-CAGGGGACGCACCAAGG-3'), 0.4 M different reverse primers (K-RP- #1: 5'-ATCACGCAGCACGCGGGTCATGG-3'; K-RP- #2: 5'-CGATG TCAGCACGCGGGTCATGG-3'; K-RP- #3: 5'-TTAGGCCAGCACGCG GGTCATGG-3'), 0.2 mM dNTP, 1U Taq polymerase (Takara, Japan). The PCR was performed using the following conditions: 94 °C for 60s, followed by 94°C for 30s, 65 °C for 30s, and a final extension of 60s at 72 °C. The PCR cycle numbers of the three groups were set as 10, 15, 20, and 25, respectively and the cycle number was taken when all of the three groups presented positive PCR bands. The PCR products were separated by 5% agarose gel electrophoresis. The aptamers in the PCR-generated products were sequenced, and modified with Indole-dU (W), Phenol-dU (Y), and Amine-dU(X) after site positioning using AM Cloud Intelligent Software (AM Biotech. Co. Ltd., USA). During synthesis, the four aptamers were simultaneously biotinylated.

Optimization of WH3Ag concentration binding to aptamers

A concentration of 1.56 μ g/mL of bovine serum albumin (BSA) and 1.56 μ g/mL of recombinant dense granule protein 15 of *T.gondii* (rGRA15, previously prepared in laboratory) [20] were used as the negative controls for the determination of affinity of aptamers to n-SAG1 protein of *Toxoplasma*. The WH3 strain

parasites were frozen and thawed 5 times in liquid nitrogen and sonicated at 3s intervals at 20W. The sonicated lysates were centrifuged at 5000rpm for 20min and the supernatants served as WH3Ag. The WH3Ag was serially diluted to 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/mL in 0.1M Na₂CO₃ and NaHCO₃ (pH9.6). After washing with 0.01 M PBS-T for five times, blocking buffer (0.5% BSA-PBS, pH 7.2) was added to each well and incubated at 37 °C for 2h. The plate was washed three times with PBS-T (pH 7.4), 100 nM biotinylated aptamers were added to each well and incubated at 37 °C for 1h. Then, the plates were washed three times with PBS-T and incubated with 100 µL of streptavidin diluted to 1:10 000 and 1:15000, which had been conjugated to HRP, at 37°C for 1h. The plates were washed with PBS-T five times and tetramethylbenzidine (TMB) was added and allowed to stand for 15 min. The color development was terminated using stop solution (H₂SO₄) and the absorbance at 450 nm was measured. The optimal concentration of WH3Ag was defined as 1.56 µg/ mL.

Binding affinity of the aptamers to n-SAG1 in WH3Ag

To detect aptamers affinity to the n-SAG1, a 96-well plate was coated with 1.56 µg/mL WH3Ag and incubated at 4 °C for overnight. The four aptamers were diluted at 0.00, 1.56, 3.125, 6.25, 12.5, 25, 50, 100, and 200 nM, respectively. After washing, 100 µL HRP-streptavidin (in dilution of 1:10 000, Shenggong Biol Co, Ltd., China) was added to each well and incubated at 37 °C for 1h, the plate was washed five times with PBS-T, and TMB was added. The plate was allowed to stand for 15min. The affinity of the four aptamers to WH3Ag was analyzed. The examinations of all samples were performed in triplicate and the OD values were measured at a wavelength of 450 nm (OD450).

Optimization of test performance and determination of cut-off value

A 96-well plate was coated with a mouse serum of acute *Toxoplasma* infection which were serially diluted from 1:5 to 1:80 for optimization of test dilution. A negative control was set up in parallel. After incubation for 2h at 37 °C, the plate was incubated at 4 °C for 12h. After 5 times of washing, 1.56 nM aptamer-2 was added to each well and incubated at 37 °C for 1h. After washing, the HRP-labeled streptavidin with a dilution of 1:10 000 was added and incubated at 37 °C for 1h. After washing as before, TMB was added after washing and allowed to stand in the dark for 15min, and then 50µL stop solution was added to each well. The specimens of all experimental groups were repeated three times and the OD values were measured at a wavelength of 450 nm. The dilution at the concentration of 1:10 presented the highest ratio of P/N (positive/negative). The optimal dilution in 1:10 for mouse sera was used and the OD450 value of 20 serum samples of normal mice was measured. The cut-off value was determined by mean±2SD and was used in the subsequent tests of mouse sera under optimized experimental conditions.

Direct enzyme-linked aptamer assay (DELAA) for human serum detection

Twenty seronegative human sera (neither IgG nor IgM against *T.gondii* examined by the commercial ELISA kit (NA Multi-Lyte ToRCH Zeus Biotech Co. Ltd, China) from the hospital were tested to calculate the threshold value by DELAA. Serum samples of 50 cases of acute *Toxoplasma* infection (with positive

IgM but negative IgG against *T.gondii*) were collected based on screening of suspected individuals with the ELISA kit. Among them, fifteen positive and 35 negative samples that had been confirmed by PCR for detection of *Toxoplasma* DNAs (Kanglang Biotech Co. Ltd, China) were re-examined with the commercial kit of *Toxoplasma* CAg-ELISA for detection of *Toxoplasma* circulating antigens (Combined Company, Shenzhen, China) [21] and DELAA, respectively, to detect circulating antigens or n-SAG1 of *T.gondii*. The serum samples were examined in triplicate and three tests were performed per sample.

Statistical analysis

Data were subject to normal distribution and statistically described as mean \pm standard deviation, and differences between groups were compared using one-way ANOVA. The chi-square test was used to compare the sensitivity and specificity of *Toxoplasma* CAg-ELISA and DELAA ($p < 0.05$ or $p < 0.01$ indicating statistical significance). All analyses and graphing were performed using GraphPad-Prism software.

Results

Expression and identification of r-SAG1

The centrifuged supernatants and sediments of dissolved *E.coli* BL-21 in PBS were subjected to SDS-PAGE. The results showed that r-SAG1 protein was mostly insoluble (Fig. 1A), but it became soluble when the buffer was replaced with 0.01 M Tris-Cl in PBS (Fig. 1B). The r-SAG1 protein was purified from the supernatants by using nickel column, reaching a purity of more than 90% (Fig. 1C). Subsequently, the immune activity of r-SAG1 was identified by Western blotting (Fig. 1D). The number of biotins labeled with one r-SAG1 molecule (d) was calculated and $A_{500 \text{ nm}}$ was determined. The absorbance of the control group was $A_{500}(1.1)$ and that of the protein group was $A'_{500}(0.22)$. The formula is as follows: $d = (\Delta_{500} \times M) / (3400 \times A)$ (A , r-SAG1 concentration: 0.5 mg/mL; M , protein molecular weight: 30 000; $\Delta_{500} = (0.9 \times A_{500}) - A'_{500}$). The results showed that one r-SAG1 molecule could be successfully labeled with 13.59 biotins on average.

Screening Of Ssdna Aptamers Against R-sag1

The ssDNA aptamers binding to r-SAG1 protein were obtained by SELEX technology. The Fig. 2A showed that 25 cycles of PCR amplification gave rise to an excellent harvest of aptamers in the three groups of initial solution (IS) control, 100 nM protein, and SMps positive control. The PCR-generated aptamers of the three groups were sequenced with Next Generation Sequencing. The modifications of aptamer oligonucleotides with Indole-dU (W), Phenol-dU (Y), and Amine-dU (X) were found to be successful when analyzed with the Cloud Intelligence Software (AM-Biotech, USA) (Fig. 2B).

Tests of optimal concentration of n-SAG1 and affinity of the aptamers

Since the aptamers were screened using the r-SAG1 protein, to determine the efficacy of the r-SAG1-recognized aptamers for detection of n-SAG1, we measured the optimal concentration of WH3Ag containing the n-SAG1 and its ability to bind the aptamers. The results showed that WH3Ag at a concentration of 1.56 µg/mL for plate coating could be clearly recognized by the aptamers in DELAA compared with the r-SAG1-positive control (Fig. 3A) and the optimal dilution of HRP-streptavidin was 1:10 000 (Fig. 3B). The affinity of the four aptamer molecules to n-SAG1 was then determined on the plate coated with WH3Ag (Fig. 4A). Furthermore, the absorbance values increased with the aptamer concentrations increased ($p = 0.001$; Spearman correlation test). Experimental absorbance and concentration data were used to calculate the dissociation constant (K_d). By using the non-linear regression equation $y = (B_{max} \times x) / (x + K_d)$, where B_{max} is the maximal affinity and K_d is the concentration of ligand required to reach half-maximal affinity, we observed that the aptamer with higher affinity against the original could obtain a lower K_d value. The K_d values of aptamer-1, aptamer-2, aptamer-3, and aptamer-4 were 56.24 ± 13.23 , 41.57 ± 9.74 , 58.98 ± 13.56 , and 42.68 ± 11.90 , respectively, suggesting that aptamer-2 has the highest affinity in the test and is suitable in DELAA for detection of n-SAG1 in the subsequent measurements ($p < 0.05$) (Fig. 4B).

Detection Of N-sag1 In Mouse Sera By Delaa

In view of the highest avidity of aptamer-2 to n-SAG1, we further tested its capacity to differentiate between positive and negative mouse sera, and the optimal concentration of the pooled sera to be detected in DELAA system. We noted that the ratio of the positive to negative sera significantly increased when the serum dilution was higher than 1:10 (Fig. 5A). In addition, the positive threshold was determined based on negative mean value and standard deviation (SD) in 25 sera of normal mice. The cut-off value was found to be 0.38 (mean + 2SD).

Under the optimized conditions, serum samples obtained from the mice infected with the virulent WH3 strain parasite on different days of infection were analyzed using DELAA in comparison with uninfected controls. The results showed that the mice presented positive n-SAG1 on day 3 and gradually reached the highest OD value on day 7 after infection (Fig. 5B). Expectedly, the OD values varied in mice infected with the low virulent and cyst-forming WH6 strain based on the parasite loads ($p \leq 0.05$), reaching a high level from day 5 to 7, and remarkably decreased on day 14 postinfection (Fig. 5C) ($p \leq 0.001$). However, no significant difference of OD450 values was noted in dosages *via* gavage on the same day of infection ($p > 0.05$).

The n-SAG1 detection by DELAA in human sera of *T.gondii* infection

Twenty human serum samples, which are negative for infection tested by *Toxoplasma*-PCR test kit, were examined to determine diagnostic threshold of DELAA. The cut-off value was found to be 0.46 (mean + 2SD). Finally, 15 positive and 35 negative sera which had been confirmed by the *Toxoplasma* PCR test kit were re-examined with both DELAA and *Toxoplasma* CAg-ELISA simultaneously. The results revealed that the sensitivity of DELAA was 93.33% (14/15) and the specificity was 94.29% (33/35), with the accuracy

of 94.00% (47/50), the positive predictive value of 87.50% (14/16), and negative predictive value of 97.06% (33/34) ($\chi^2 = 37.045$, $p < 0.05$). A comparison of *Toxoplasma* CAg-ELISA with *Toxoplasma* PCR showed the sensitivity of 73.33% (11/15) and specificity of 82.86% (29/35), with the accuracy of 80.00% (40/50), the positive predictive value of 64.71% (11/17) and the negative predictive value of 87.88% (29/33) ($\chi^2 = 14.774$, $p < 0.05$), respectively (Table 1). The data indicate a better efficacy of DELAA than *Toxoplasma* CAg-ELISA in sensitivity and specificity in diagnosis of acute *Toxoplasma* infection (Table 2).

Table 1
A comparison of DELAA and *Toxoplasma* CAg-ELISA using PCR-positive and -negative human sera for diagnosis of *T.gondii* infection(n = 50)

		DELAA*		<i>Toxoplasma</i> CAg- ELISA**	
		+	-	+	-
PCR	+	14	1	11	4
	-	2	33	6	29

* $\chi^2 = 37.045$, $p < 0.05$; ** $\chi^2 = 14.774$, $p < 0.05$

Table 2
A comparison of DELAA with *Toxoplasma* CAg-ELISA using IgM-positive and IgG-negative human sera (n = 50) for diagnosis of *T.gondii* infection

		DELAA	
		+	-
<i>Toxoplasma</i> CAg- ELISA	+	13	2
	-	6	29

$\chi^2 = 21.5417$, $p < 0.05$

Discussion

The majority of human chronic *Toxoplasma* infection are benign. Nevertheless, a generalized infection probably occurs in a small percentage of cases with immune deficiency, with the clinical manifestations ranging from mild to deleterious results. Diagnosis of toxoplasmosis is seldom made by recovery of organisms because the parasite dwells in tissues. Cell culture is frequently used instead of animals for demonstration of the infection. Additionally, a polymerase chain reaction (PCR) test has been routinely applied to detection of the *Toxoplasma*-derived DNAs in samples of blood, amniotic fluids and diseased tissues in acute and reactivated toxoplasmosis [22]. The laboratory tests of value are the various serological procedures that have been employed for measurements of antibodies against *T.gondii*, and

have been widely used not only for individual diagnosis but for epidemiological survey. The conventional diagnosis includes detection of IgG, IgM and/or IgA antibodies against *Toxoplasma* in sera of human or animals using a variety of methods. These antibody-based techniques, however, are not always so relevant in defining acute or reactivated toxoplasmosis, and high titers of antibodies may be just indicative of the active disease (positive IgM or low avidity of IgG) or higher risk to develop it (permanently positive IgG) [23, 24]. Treatment is not usually recommended in the individuals of asymptomatic latent infection with only positive anti-*Toxoplasma* IgG antibodies.

Alternatively, a variety of molecules are actively secreted/excreted by *Toxoplasma* parasite on the time of and during tachyzoite invasion of host cells and constitute an essential part of circulating antigens (CAs) [25]. During reactivation of the tissue-dwelling quiescent cysts, numerous tachyzoites, together with abundant excretory/secretory antigens (ESAs), are released, which play a crucial role in invasion process and induction of host immune response against the parasite [26, 27]. These CAs/ESAs are a panel of good candidates for investigation into new diagnostic markers [28], and detection of these antigenic substances using specific antibodies have been shown to be considerably useful. Among them, surface antigen 1 (SAG1), previously named as p30, is a predominant molecule on the membrane of tachyzoites [29, 30]. SAG1 is a stage-specific antigen presented only in tachyzoites with 336 amino acids and six disulfide bonds, and has high degree of conformation and conservation in structure among virulent strains of *T. gondii*. These properties make it an attractive antigen for diagnostics and vaccine development [11]. Having excellent immune features, recombinant SAG1 has been the most studied antigen of *Toxoplasma* for development of antibody-based serological tests and vaccine [31, 32]. A previous study by Letillois and collaborators showed that recombinant SAG1 may be used as a marker of reactivated toxoplasmosis in HIV infected patients [10]. Few methods, however, are available as routine tools for direct detection of *T. gondii* SAG1 protein in sera although the anti-SAG1-based ELISA, or SAG1-based molecular diagnostic PCR, has been used for diagnosis of toxoplasmosis [10, 11].

The SELEX (systematic evolution of ligands by exponential enrichment) technique was generated by using reiterative *in vitro* selection for high-affinity and -specificity oligonucleotide ligands (aptamers) against almost any kind of molecules that are of biological or therapeutic interest [33]. Aptamers provide a powerful tool which is not merely useful for identification of novel diagnostic markers but for interference with the duplication of the pathogenic agents, and thus have been widely used as biorecognition probes against human pathogens and/or their toxins [34–36].

In this study, we generated ssDNA aptamers against recombinant *Toxoplasma* SAG1 (r-SAG1) by the SELEX technology. Four aptamers that recognize and target the natural *Toxoplasma* SAG1 (n-SAG1) were screened and enriched, and a direct enzyme-linked aptamer assay (DELAA) was primarily established and evaluated using r-SAG1 and total lysates of *T. gondii* WH3 strain (WH3Ag), a virulent strain of type Chinese 1 which dominates in animals and human of China [17]. Sera collected from the suspected individuals with *Toxoplasma* infection and those who were seropositive for anti-*Toxoplasma* IgM antibodies were tested by DELAA at optimized conditions. We found that aptamer-2, among the four candidates, was the appropriate detective probe for tests due to its high affinity to n-SAG1 when

compared with the others (Fig. 4). The OD values of whole cell lysates of WH3 strain parasite diluted to 1.56 µg/mL could be clearly differentiated between the positive control of purified r-SAG1 and BSA negative control (Fig. 3). The mice with 3 days infection of virulent strain of *T.gondii* and 7 days infection of low virulent strain became detectable by DELAA. Furthermore, 14 of 15 samples of *Toxoplasma* infection, that had been defined by PCR, presented positive results when aptamer-2 was used as the bioprobe, with 93.33 % sensitivity and 94.29% specificity, indicating that aptamer-2 based DELAA has a higher test performance compared to the antibody based *Toxoplasma* CAg-ELISA for human diagnosis of toxoplasmosis ($p \leq 0.05$), and may provide a novel strategy for detection of *T.gondii* SAG1 protein. Thus DELAA could aid in the point-of-care diagnosis of acute or reactivated toxoplasmosis.

Aptamers offer several advantages over their antibody counterparts although they share some similarities in terms of avidity and specificity to antigen molecules. For instance, unlike antibody generation which requires the use of live animals and immunogenic and nontoxic target molecules to elicit mouse antibody response, animals or cells are not involved in the production of aptamers due to application of an *in vitro* pure chemical process in selection. Additionally, ssDNA aptamers are quite stable at ambient temperature, whereas antibodies require refrigeration to avoid degradation. Finally, aptamers possess significant features such as small size (~ 30–80 nucleotides), easy modification, high efficient penetration into tissues, low cost and time-saving of production, and may provide a high potential in development of non-protein-based diagnostic as well as bio-therapeutic agents, not only for major infectious diseases but also for cancers [37].

Aptamers-based method has been widely employed in the detection of various bacterial and viral infections [36]. In the last decade, considerable achievements were made using aptamer-based assay in diagnoses of some of the most challenging protozoan parasites infecting humans in laboratory settings [14]. Lou Y *et al.* selected two specific aptamers of TGA6 and TGA7 targeting anti-*Toxoplasma* IgG and developed a quantum dots-labeled dual aptasensor (Q-DAS), which achieved as high as 94.8% sensitivity and 95.7% specificity by reading the labeled fluorescence when compared with antibody-based immunoassay [15]. Recently, an enzyme-linked aptamer assay (ELAA) with newly selected aptamers (AP001 and AP002) against ROP18 protein, a crucial virulence-associated factor secreted by *Toxoplasma* tachyzoites, were established and used to evaluate total protein from *T. gondii* RH strain and recombinant ROP18 [16]. The ELAA demonstrated higher affinity and specificity to RH strain antigen and rROP18. Detection limit of rROP18 in sera reached a concentration as low as 1.56 µg/mL. A significant association between positive ELAA and severe congenital toxoplasmosis was noted in seropositive and control human samples, which is consistent to our present investigation. Moreover, being the major surface protein of *Toxoplasma* tachyzoite, SAG1 is believed to be the most reliable marker targeted by its recognition aptamers. Further work is needed to explore the probability of cross-reaction of the aptamer to the SAG homologs from other apicomplexan parasites.

In conclusion, we developed a direct enzyme-linked aptamer assay (DELAA) with an aptamer recognition probe for detection of the native SAG1 protein of *T.gondii* tachyzoite, which has high sensitivity, specificity and reproducibility. The novel aptamer-based technique is considered as an efficient and

promising method for diagnosis of acute and reactivated toxoplasmosis, a disease of significance in immunocompromised patients, pregnant women as well as animal husbandry production.

Declarations

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Availability of data and materials

All datasets used and/or analyzed in the current study are included in this published article and its additional files.

Ethics approval and consent to participate

The Ethical Permission was obtained from Clinical Trial Ethics Committee of the Second Hospital of Hefei, Anhui (permission No. 2020-Ke-054∩), and the Informed Consent was given by each of the individuals who received the tests in this work.

Authors' contributions

WW and JS designed the work; XS,WC and AB performed the experiments and collected the data; WC, CW and QW collected the sera and analyzed the data; XS and WC drafted the article; LZ, QT, LY, and JD helped for screening of the aptamers; JS, YX and OC did critical revision of the article. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Written informed consent for publication of their clinical details and/or clinical images was obtained from the patients or guardians of the patient.

References

1. Waldman BS, Schwarz D, Wadsworth IIMH, Saeij JP, Shalek AK, Lourido S. Identification of a master regulator of differentiation in *Toxoplasma*. *Cell*. 2020;180:359–72.
2. Tuazon CU. Toxoplasmosis in AIDS patients. *J Antimicrob Chemother*. 1989;23(Suppl A):77–82.
3. Kerl K, Ehlert K, Brentrup A, Schiborr M, Keyvani K, Becker K, et al. Cerebral toxoplasmosis in an adolescent post allogeneic hematopoietic stem cell transplantation: successful outcome by antiprotozoal chemotherapy and CD4 + T-lymphocyte recovery. *Transpl Infect Dis*. 2015;17:119–24.
4. Pulivarthi S, Reshi RA, McGary CT, Gurram MK. Cerebral toxoplasmosis in a patient on methotrexate and infliximab for rheumatoid arthritis. *Intern Med*. 2015;54:1433–6.
5. Dard C, Marty P, Brenier-Pinchart MP, Garnaud C, Fricker-Hidalgo H, Pelloux H, et al. Management of toxoplasmosis in transplant recipients: an update. *Expert Rev Anti Infect Ther*. 2018;16:447–60.
6. Tomasoni LR, Messina G, Genco F, Scudeller L, Prestia M, Spinoni V, et al. Risk of congenital toxoplasmosis in women with low or indeterminate anti-*Toxoplasma* IgG avidity index in the first trimester of pregnancy: an observational retrospective study. *Clin Microbiol Infect*. 2019; 25:761 e9-761 e13.
7. Thuy Doan NR, Acharya BA, Pinsky MK, Sahoo ED, Chow N, Banaei, et al. Metagenomic DNA sequencing for the diagnosis of intraocular infections *Ophthalmology*. 2017;124(8):1247–8.
8. Robert-Gangneux F, Darde ML. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin Microbiol Rev*. 2012;25:264–96.
9. Villavedra M, Rampoldi C, Carol H, Baz A, Battistoni JJ, Nieto A. Identification of circulating antigens, including an immunoglobulin binding protein, from *Toxoplasma gondii* tissue cyst and tachyzoites in murine toxoplasmosis. *Int J Parasitol*. 2001;31:21–8.
10. Letillois MF, Laigle V, Santoro F, Micoud M, Chumpitazi BF. *Toxoplasma gondii* surface antigen-1 in sera of HIV-infected patients as an indicator of reactivated toxoplasmosis. *Eur J Clin Microbiol Infect Dis*. 1995;14:899–903.
11. Wang Y, Yin H. Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*. *Parasit Vectors*. 2014;7:180.
12. Yu H, Huang B, Zhuo X, Chen X, Du A. Evaluation of a real-time PCR assay based on the single-copy SAG1 gene for the detection of *Toxoplasma gondii*. *Vet Parasitol*. 2013;197:670–3.
13. Robertson DL, Joyce GF. Selection in vitro of an RNA enzyme that specifically cleaves single-stranded DNA. *Nature*. 1990;344:467–8.
14. Li HY, Jia WN, Li XY, Zhang L, Liu C, Wu J. Advances in detection of infectious agents by aptamer-based technologies. *Emerg Microbes Infect*. 2020;9:1671–81.
15. Luo Y, Liu X, Jiang T, Liao P, Fu W. Dual-aptamer-based biosensing of *Toxoplasma* antibody. *Anal Chem*. 2013;85:8354–60.
16. Vargas-Montes M, Cardona N, Moncada DM, Molina DA, Zhang Y, Gomez-Marin JE. Enzyme-linked aptamer assay (ELAA) for detection of *Toxoplasma* ROP18 protein in human serum. *Front Cell Infect*

Microbiol. 2019;9:386.

17. Li M, Mo X-W, Wang L, Chen H, Luo Q-L, Wen H-Q, et al. Phylogeny and virulence divergency analyses of *Toxoplasma gondii* isolates from China. *Parasit Vectors*. 2014;7:133.
18. Wang L, Chen H, Liu D, Huo X, Gao J, Song X, et al. Genotypes and mouse virulence of *Toxoplasma gondii* isolates from animals and humans in China. *Plos One*. 2013;8(1):e53483.
19. Quan Wang W, Jiang Y-J, Chen C-Y, Liu. Jin-lei Shi, and Xin-tong Li. Prevalence of *Toxoplasma gondii* antibodies, circulating antigens and DNA in stray cats in Shanghai. *ChinaParasites Vectors*. 2012;5:190.
20. Xie Y, Wen H, Yan K, Wang S, Wang X, Chen J, et al. *Toxoplasma gondii* GRA15_{II} effector-induced M1 cells ameliorate liver fibrosis in mice infected with *Schistosomiasis japonica*. *Cell Mol Immunol*. 2018;15:120–34.
21. Quan Wang W, Jiang Y-J, Chen C-Y, Liu. Jin-lei Shi and Xin-tong Li. Prevalence of *Toxoplasma gondii* antibodies, circulating antigens and DNA in stray cats in Shanghai. *ChinaParasites Vectors*. 2012;5:190.
22. Quan Liu ZD, Wang S, Huang X, Zhu. Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. *Parasit Vectors*. 2015;8:292.
23. Colombo FA, Vidal JE, Penalva de Oliveira AC, Hernandez AV, Bonasser-Filho F, Nogueira RS, et al. Diagnosis of cerebral toxoplasmosis in AIDS patients in Brazil: importance of molecular and immunological methods using peripheral blood samples. *J Clin Microbiol*. 2005;43:5044–7.
24. Vidal JE, Diaz AV, de Oliveira AC, Dauar RF, Colombo FA, Pereira-Chiocola VL. Importance of high IgG anti-*Toxoplasma gondii* titers and PCR detection of *T. gondii* DNA in peripheral blood samples for the diagnosis of AIDS-related cerebral toxoplasmosis: a case-control study. *Braz J Infect Dis*. 2011;15:356–9.
25. Ahn MH, Son HJ, Leem MH, Min DY. Antigen analysis of *Toxoplasma gondii* lysate and excretory-secretory materials by enzyme-linked immunoelectrotransfer blot (EITB). *Korean J Parasitol*. 1994;32:249–57.
26. Prigione I, Facchetti P, Lecordier L, Deslee D, Chiesa S, Cesbron-Delauw MF, et al. T cell clones raised from chronically infected healthy humans by stimulation with *Toxoplasma gondii* excretory-secretory antigens cross-react with live tachyzoites: characterization of the fine antigenic specificity of the clones and implications for vaccine development. *J Immunol*. 2000;164:3741–8.
27. Saadatnia G, Mohamed Z, Ghaffarifar F, Osman E, Moghadam ZK, Noordin R. *Toxoplasma gondii* excretory and secretory antigenic proteins of diagnostic potential. *APMIS*. 2012;120:47–55.
28. Hafid J, Raberin H, Pozzetto B, Akono ZY, Sung RT, Jana M, et al. Comparison of excretory/secretory and circulating antigens of *Toxoplasma gondii* by enzyme immunoassay and immunoblotting. *Int J Parasitol*. 1992;22:1083–8.
29. Khan IA, Ely KH, Kasper LH. A purified parasite antigen (p30) mediates CD8 + T cell immunity against fatal *Toxoplasma gondii* infection in mice. *J Immunol*. 1991;147:3501–6.

30. Burg JL, Perelman D, Kasper LH, Ware PL, Boothroyd JC. Molecular analysis of the gene encoding the major surface antigen of *Toxoplasma gondii*. *J Immunol*. 1988;141:3584–91.
31. Wu K, Chen XG, Li H, Yan H, Yang PL, Lun ZR, et al. Diagnosis of human toxoplasmosis by using the recombinant truncated surface antigen 1 of *Toxoplasma gondii*. *Diagn Microbiol Infect Dis*. 2009;64:261–6.
32. Sun X, Lu H, Jia B, Chang Z, Peng S, Yin J, et al. A comparative study of *Toxoplasma gondii* seroprevalence in three healthy Chinese populations detected using native and recombinant antigens. *Parasit Vectors*. 2013;6:241.
33. Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. *Nature*. 1990;346:818–22.
34. Pan Q, Luo F, Liu M, Zhang XL. Oligonucleotide aptamers: promising and powerful diagnostic and therapeutic tools for infectious diseases. *J Infect*. 2018;77:83–98.
35. Zimbres FM, Tarnok A, Ulrich H, Wrenger C. Aptamers: novel molecules as diagnostic markers in bacterial and viral infections. *Biomed Res Int*. 2013; 731516.
36. Alizadeh N, Memar MY, Mehramuz B, Abibiglou SS, Hemmati F, Samadi Kafil H. Current advances in aptamer-assisted technologies for detecting bacterial and fungal toxins. *J Appl Microbiol*. 2018;124:644–51.
37. Zhou G, Latchoumanin O, Hebbard L, Duan W, Liddle C, George J, et al. Aptamers as targeting ligands and therapeutic molecules for overcoming drug resistance in cancers. *Adv Drug Deliv Rev*. 2018;134:107–21.

Figures

Fig. 1

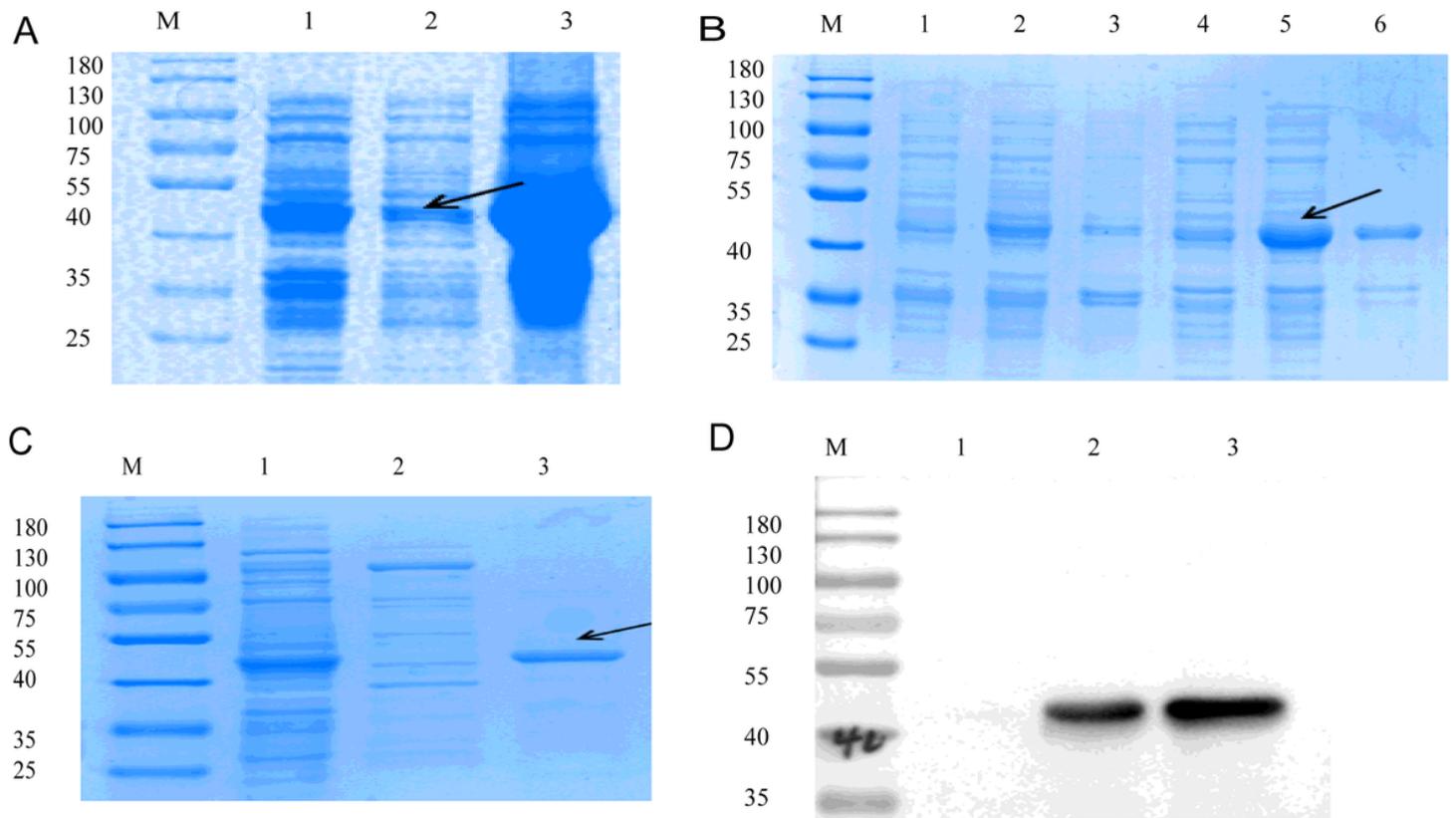


Figure 1

Soluble expression and identification of Toxoplasma r-SAG1 in supernatants of sonicated lysates of E.coli after centrifugation; M, markers; 1, E.coli lysates induced by IPTG for 5 h; 2, supernatants of lysate centrifugation; 3, sediments of lysate centrifugation; (B) soluble r-SAG1 in supernatants of the E.coli lysates in 0.01M Tris-Cl in stead of PBS(1, lysate control of E.coli; 2 and 3, supernatants and sediments of lysate control of E.coli; 4, total E.coli lysates containing r-SAG1; 5 and 6, supernatants and sediments of the lysates following treatment of 0.01M Tris-Cl in PBS); (C) r-SAG1 protein purified through nickel column (M, markers; 1, IPTG-induced supernatants of E.coli lysates; 2, impure protein of E.coli lysates; and 3, supernatant purification for r-SAG1 by nickel column); and (D) r-SAG1 recognized by rabbit polyclonal IgG antibodies against T.gondii. (M, markers; 1, supernatants of blank E.coli lysate control; 2, supernatants of lysate centrifugation; 3, supernatant purification for r-SAG1 by nickel column).

Fig. 2

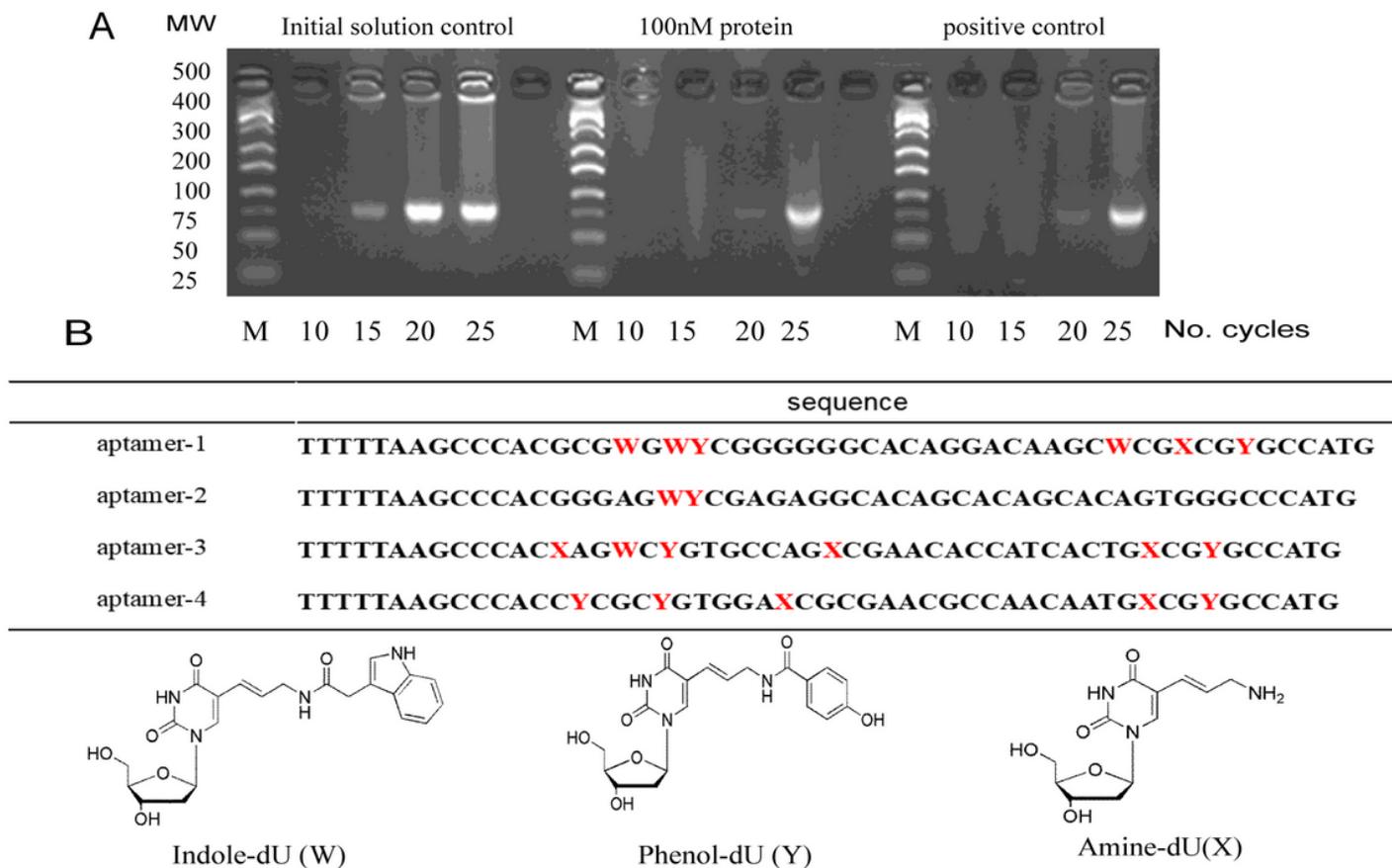


Figure 2

Optimization of aptamers gene amplification by PCR (A) Twenty-five cycles of PCR amplification resulted in abundant production of aptamers; and (B) Four aptamers with given conformations were screened and identified by Next Generation Sequencing.

Fig. 3

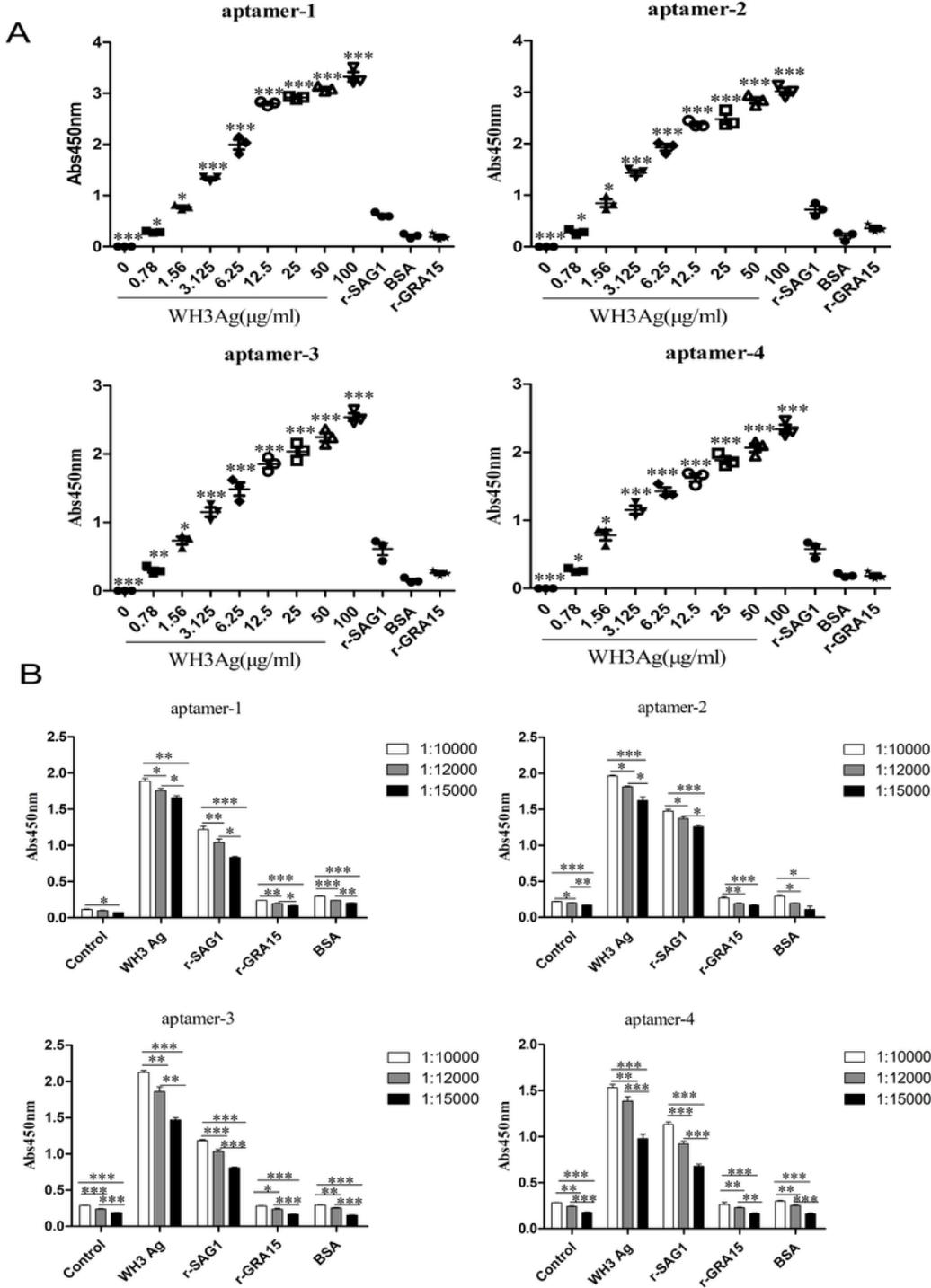
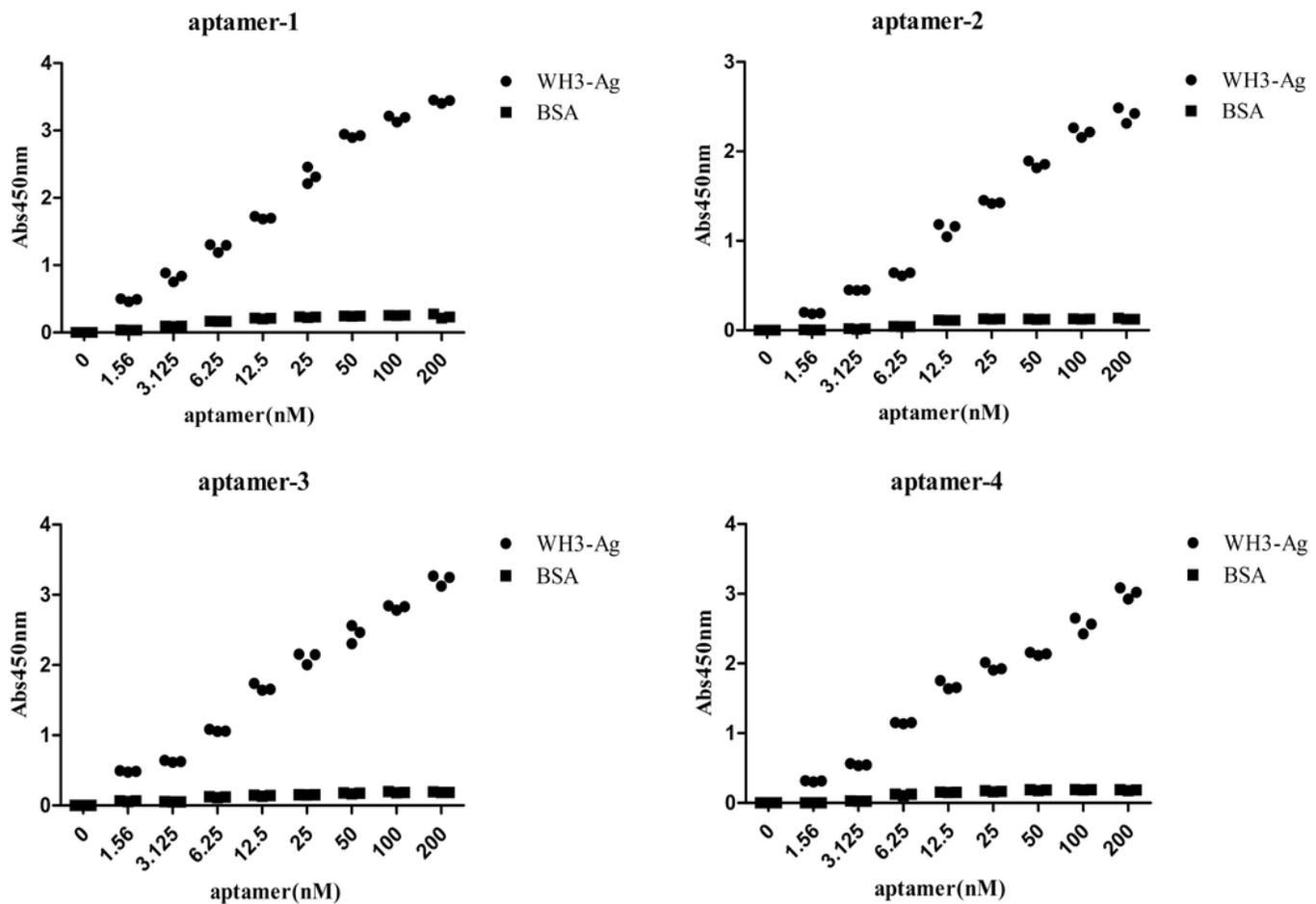


Figure 3

Definition of optimal dilution of the n-SAG1 in WH3Ag detected by DELAA (A) The WH3Ag was serially diluted from 200 µg/mL to 0.78 µg/mL. The r-SAG1 and BSA at 1.56 µg/mL were taken as positive and negative controls. It shows that a concentration of 1.56 µg/mL WH3Ag gave rise to the best performance; (B) the optimal dilution of HRP-streptavidin was 1:10 000 (*p<0.05;**p<0.01;***p<0.001).

Fig. 4

A



B

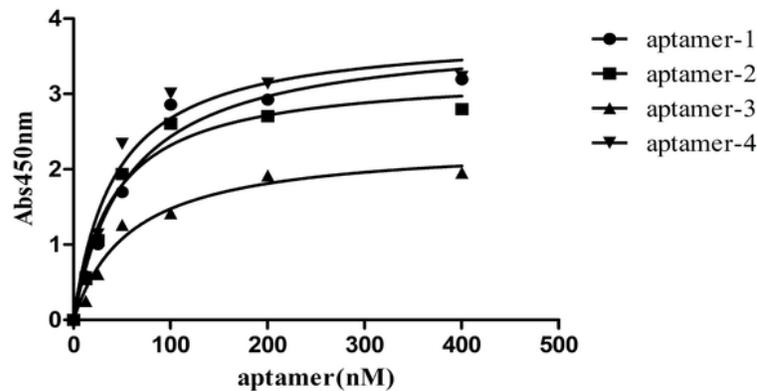
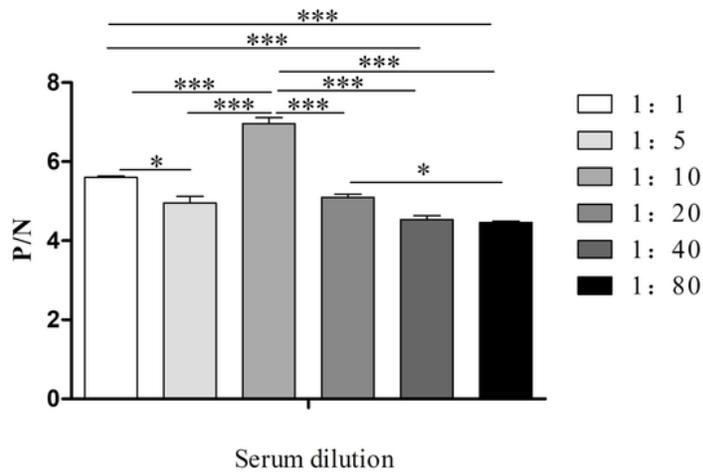


Figure 4

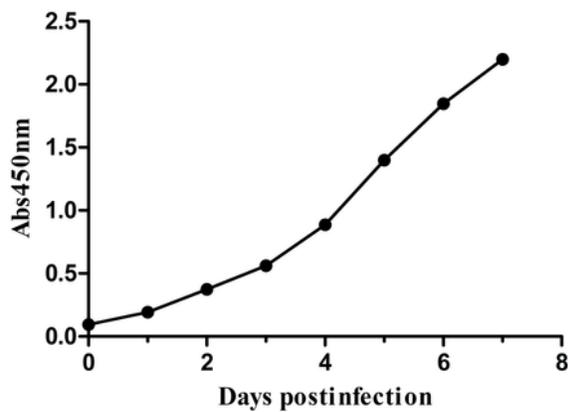
Affinity of the four aptamers binding to the n-SAG1 in WH3Ag tested by DELAA (A) A serial dilution of the aptamers from 200 $\mu\text{g}/\text{mL}$ to 1.56 $\mu\text{g}/\text{mL}$ was used, and BSA served as negative control ($p < 0.001$, Spearman correlation test); and (B) Nonlinear regression analysis of $y = (B_{\text{max}} \times x) / (x + K_d)$ indicates that aptamer-2 has a smallest K_d value (41.57 ± 9.70), suggesting a highest avidity to n-SAG1 compared with the other three aptamers ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$).

Fig. 5

A



B



C

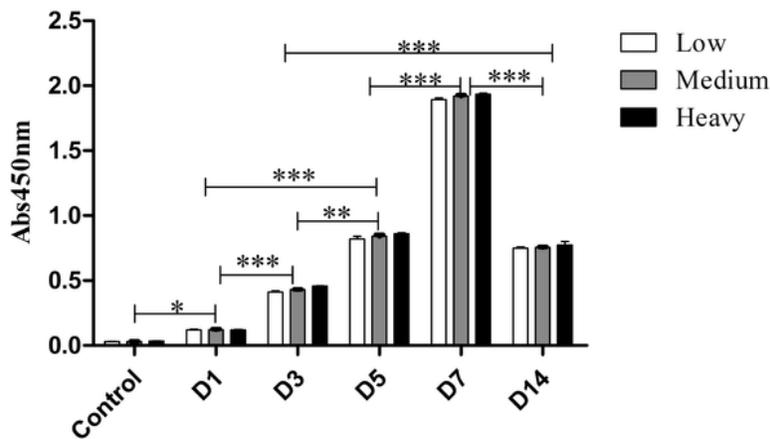


Figure 5

Optimization of working dilution of mouse sera and HRP-avidin in DELAA for detection of n-SAG1. (A) The ratio of OD value using pooled sera of *Toxoplasma*-infected mice to normal animals decreased notably when the dilution was higher than 1:10; Positive/Negative (P/N) was statistically significant under this condition. (B) Mice infected with virulent WH3 strain of *T.gondii* presented a strongly positive OD value (OD_{450 nm}: 0.63, cut-off value: 0.38) when detected at different time postinfection; (C) Mice

were infected via gavage with different doses of low virulent and cyst-forming WH6 strain of *T.gondii*. Thirty mice in each of the three groups were individually infected with 30 (low load group), 60 (medium load group), and 90 cysts (heavy load group), respectively. Sera were collected and detected at various time postinfection with DELAA. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Supplementary Files

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