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

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Research

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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 quiescent cysts and latent infection of the parasite. As a major surface antigen of *T.gondii* tachyzoites, SAG1 is a key marker for laboratory diagnosis. However, at present, there are no methods available for SAG1 detection using aptamer-based technology.

**Methods:** Recombinant truncated SAG1 (r-tSAG1) of *Toxoplasma* WH3 strain (type Chinese 1) was prokaryotically expressed and subjected to the synthetic oligonucleotide library for selection of nucleic acid aptamer which targets the r-tSAG1, with systematic evolution of ligands by exponential enrichment (SELEX) strategy. The screened specific aptamer-2 was used in direct enzyme-linked aptamer assay (DELAA) to detect native SAG1 obtained from tachyzoite lysates, mouse sera of acute infection, and human sera that had been verified to be positive for *Toxo*DNAs by PCR amplification.

**Results:** The soluble r-tSAG1 protein was obtained from *E.coli* lysates by using 0.01M Tris-Cl in PBS, and was purified and identified by immunoblotting, and then labelled with biotin. The screened aptamers were amplified by PCR, followed by DNA sequencing. The results showed that the aptamer-2, with the highest affinity to nSAG1 among the four aptamer candidates, has a higher specificity and sensitivity when used in detection of nSAG1 in the sera of both animals and humans when compared with the commercial *Toxoplasma* circulating antigen testing kit.

**Conclusions:** A new direct enzyme-linked aptamer assay (DELAA), with aptamer-2 as the recognition probe, was developed for detection of native SAG1 protein secreted by *T.gondii*. With increased sensitivity and specificity, stability during storage, easy and cheaper production, the aptamer-based technique is considered as an efficient method for the diagnosis of active and reactivated toxoplasmosis.

**Background**

*Toxoplasma gondii* is an obligatory intracellular apicomplexan 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. However, its recrudescence can cause severe clinical disease in the immunocompromised individuals such as those with HIV/AIDS[2], patients receiving chemotherapy[3], patients on long-term immunosuppressive treatments[4], or recipients of organ transplants[5]. The main forms of toxoplasmosis in these subjects include severe ocular, neurologic, and sometimes systemic disease. Primary infection with *Toxoplasma* during pregnancy, particularly in the first trimester, may cause stillbirths, miscarriages, or fetal abnormalities[6].

Most cases of *Toxoplasma* infections elude diagnosis due to the lack of distinct clinical manifestations, difficulty in obtaining specimens from individuals with latent infection, and simple methods used in pathogenic diagnosis. 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 sequencing. Usually, the IgG antibody test is used to determine chronic infection whereas IgM and IgA antibody tests help in the diagnosis of acute or congenital toxoplasmosis[7,8]. 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 [9]. Serological diagnosis is usually retrospective and is used to determine 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 no or low number of 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 in biomarker 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[10]. 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 and a highly immunoreactive and immunogenic antigen. It probably represents the most explored and used antigen of *T. gondii* for development of serological tests[11] and preventive vaccine due to its abundance on the cell surface, reaching 3-5% of total proteins[12]. A real-time PCR assay using a set of primers targeting the SAG1 gene showed higher sensitivity for the fast and specific detection of *T. gondii*[13]. However, little is known concerning the efficacy of the SAG1 protein detection 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)[14]. 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 without animal use and 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 protozoan parasites[15]. 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-ToxoIgG-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[16]. It has been known that rhoptry 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[17].

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 detection of SAG1 protein in sera of infected animals and humans. Here, we demonstrated that Selex-derived aptamer-2 is able to target the native SAG1 (nSAG1) of *Toxoplasma* 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-tSAG1) protein, in the direct enzyme-linked aptamer assay (DELAA) to detect the nSAG1 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. Our results showed that aptamer-2 could recognize the nSAG1 protein with high specificity, sensitivity and repeatability in measurement of either r-tSAG1 or nSAG1 when compared with rGRA15 and BSA negative controls. 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 cultivation and WH3Ag preparation**

Human foreskin fibroblasts (HFF, ATCC® SCRC-1041™) cells were cultured in DMEM medium containing 10% fetal bovine serum, 100µg/ml streptomycin and 100 U/ml penicillin. After the cells reached 85% confluency, the virulent WH3 strain tachyzoites of *T. gondii* (type Chinese 1) 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 antigen (WH3Ag). The cysts of low virulent WH6 strain (type Chinese 1) were harvested by mouse infection and cysts were collected from the brain tissues of mice.

**Animal infection and sera 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 mice were treated strictly in compliance with the Chinese National Institute of Health Guide for the Care and
Use of Laboratory Animals (permission No. AMU26-081108). All of the animal experimental procedures were performed in licensed Biosafety II Laboratory. Thirty-five mice were inoculated intraperitoneally with 100 tachyzoites suspended in 100 μL of PBS, and five mice injected with 100 μL PBS were taken as controls. Sera were collected from the animals under euthanasia on the day 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 the day 1, 3, 5, 7, and 14 post-infection. The sera were collected and stored at -80 °C for future use.

**Preparation of r-tSAG1 and its biotin-labeling in vitro**

The truncated SAG1 encoding gene (*sag1*) (forward primer: 5'- GGATCCTTCATCTCTAAATGC CCTA- 3'; reverse primer: 5'- AAGCTTCCTGCACGAAACT-3') was amplified using *Toxoplasma* cDNA as template, ligated to the vector (pET32a), and transformed into *E.coli* BL21 host cells to express r-tSAG1 protein. The host cells induced by IPTG were sonicated at 5s intervals and centrifuged at 12 000 rpm for 20 min to obtain the supernatants. The r-tSAG1 protein with 6×His tags was purified using nickel column (Millipore, USA) and used for preparation of anti-*Toxoplasma* IgG of rabbits. The r-tSAG1 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-tSAG1 (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 biotin labeling of the r-tSAG1 protein, 1.875 μL 20mM EZ-Link NHS-PEG4-Biotin (Thermo, No. 21329, USA) was added to 0.1mL 0.5mg/mL r-tSAG1 protein at 4℃ for overnight, ensuring that one r-tSAG1 protein could be labeled with 3-5 biotin molecules. The r-tSAG1 protein was dialyzed in PBS to remove excessive NHS-PEG4-Biotin, and NHS-PEG4-Biotin-labeled r-tSAG1 was used for subsequent aptamers screening.

**Screening of ssDNA aptamers against r-tSAG1 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 oligonucleotides were mixed with 2 mL buffer A (containing 0.5nM BSA and 1nM MgCl₂) 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 ℃ for 30 min, in order to remove the aptamers that could bind to M-280 SMps. Next, the biotin-r-tSAG1 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 labelled r-tSAG1-aptamer complex were mixed with 50μL 1nM NaOH and incubated at 65℃ for 30 min. Then 40μL 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 15μL each (#1 initial solution control: 135μL buffer A; #2 100nM protein: 30μL r-tSAG1 and 100μL buffer A; #3 positive SMps control: 130μL 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 30 min. The groups #2 and #3 were placed on magnetic racks to separate SMps for PCR. The three groups were amplified separately by PCR containing 10μL 10×PCR buffer, 2.5mM MgCl₂, 0.4 M forward primer (K-FP: 5’-CAGGGGACGCACCAAGG-3’), 0.4 M reverse primers (K-RP- #1: 5’-ATCACGC AGCACGCGGGTCATGG-3’; K-RP- #2: 5’-CGATG TCAGCACGCGGGTCATGG-3’; K-RP- #3: 5’-TTAGGCCAG CACGCGGGTCATGG-3’), 0.2 mM dNTP, 1U Taq polymerase (Takara, Japan). The PCR was performed using the following conditions: 94℃ for 60s, followed by 94℃ for 30s, 65℃ for 30s, and a final extension of 60s at 72℃. The sequence of the aptamers in the PCR products were completed by second-generation sequencing and derived using AM Cloud Intelligent Software. During synthesis, the four aptamers were labeled with biotin (AM Biotech. Co. Ltd., USA).

Optimization of WH3Ag concentration binding to aptamers

Bovine serum albumin (BSA) and recombinant dense granule protein 15 of *T. gondii* (rGRA15, previously prepared in laboratory) [18] were used as the negative controls for the determination of affinity of aptamers to nSAG1 protein of *Toxoplasma*. The WH3 strain parasites were frozen and thawed 5 times in liquid nitrogen and sonicated at 3s intervals at 20W. The supernatants (WH3Ag) were obtained by lysate centrifugation at 5000rpm for 20min, and diluted to 1.56μg/mL, 3.125μg/mL, 6.25μg/mL, 12.5μg/mL, 25μg/mL, 50μg/mL, 100μg/mL, and 200μg/mL in 0.1M Na₂CO₃ and NaHCO₃ (pH9.6). After washing with 0.01M PBS-T for five times, blocking buffer (0.5% BSA-PBS, pH 7.2) was added to each well and incubated at 37℃ for 2h. The plate was washed three times with PBS-T (pH7.4), 100nM biotin-aptamers were added to each well and incubated at 37℃ for 1h. Then, the plates were washed three times with PBS-T and incubated with 100μL of 1:10 000 diluted streptavidin conjugated to HRP for 1h at 37℃. The plates were washed with PBS-T five times and TMB was added and allowed to stand for 15min. The color development was terminated using stop solution (H₂SO₄) and the absorbance at 450nm was measured. The optimal concentration of WH3Ag was 1.56μg/mL.

Binding affinity of the aptamers to nSAG1 in WH3Ag

To detect aptamers affinity to the nSAG1, a 96-well plate was coated with 1.56μg/mL WH3Ag and incubated at 4℃ for overnight. The four aptamers were diluted at 0nM, 1.56nM, 3.125nM, 6.25nM, 12.5nM, 25nM, 50nM, 100nM, and 200nM, respectively. After washing, 100μL streptavidin-HRP (1:10 000) (Shenggong Biol Co., Ltd., China) was added to each well and incubated at 37℃ 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 three times 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 the mouse sera of acute *Toxoplasma* infection serially diluted from 1:5 to 1:80. The negative controls were 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, 1.56nM aptamer-2 was added to each well and incubated at 37°C for 1h. After washing, the avidin-labeled HRP with a dilution of 1:10 000, 1:12 000, and 1:15 000 was added and incubated at 37°C for 1h. After washing as stated above, 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 absorbance was determined at 450nm. The OD450 value of 20 serum samples of normal mice was measured. The cut-off value was determined by mean±2SD.

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

The 20 seronegative human sera were tested to calculate the cut-off value by DELAA. Serum samples of 50 cases of acute *Toxoplasma* infection (with positive anti-ToxoIgM but negative anti-ToxoIgG) were collected based on screening suspected individuals using ELISA technique. Additionally, thirty five negative and fifteen positive samples that had been confirmed by *Toxoplasma* nucleic acid test kit (Kanglang Biotech Co. Ltd, China) were re-examined with the commercial ToxoCAg-ELISA kit (Shanghai Enzyme-linked Biotech. Co. Ltd., China) and DELAA respectively to detect circulating antigens/SAG1 of *T.gondii*. The serum samples were examined in triplicate and three tests were performed per sample. Informed written consents were obtained from all participants that accepted to participate in the study.

Statistical analysis

The data were presented as the mean ± SD and were statistically analyzed using one-way ANOVA after precheck for normal distribution and homogeneity of variances, and *p*<0.05 or *p*<0.01 indicated statistical significance. The sensitivity and specificity of ToxoCAg-ELISA were evaluated using the Chi-square test in comparison with DELAA. Analyses of experimental data and graphic production were performed using GraphPad Prism Software.

Results

Expression and identification of r-tSAG1

The centrifuged supernatants and sediments of dissolved *E.coli* BL-21 in PBS were subjected to SDS-PAGE. The results showed that r-tSAG1 protein was mostly insoluble (Fig. 1A), but it became soluble when the buffer was replaced with 0.01M Tris-Cl in PBS (Fig. 1B). The r-tSAG1 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-tSAG1 was identified by Western blotting (Fig. 1D). The number of biotin labeled with one r-tSAG1 molecule (d) was calculated and A500nm was determined. The absorbance of the control group was A500(1.1) and that of the protein group was A'500(0.22). The formula is as follows: d= (△500 x M)/(3400 x A) (A, r-tSAG1 concentration: 0.5 mg/mL; M, protein molecular weight: 30 000; △500= (0.9 x
A500) - A '500). The results showed that one r-tSAG1 could be successfully labeled with 13.59 molecules of biotin on average.

**Screening Of Ssdna Aptamers Against R-tsag1**

The ssDNA aptamers binding to r-tSAG1 protein were obtained by SELEX technology. After 25 cycles of amplification, the aptamers presence produced an excellent effect (Fig. 2A) in the three groups of initial solution(IS) control, 100 nM protein, and SMps positive control. The sequences of aptamers in PCR-generated products of the three groups were determined with the second-generation sequencing, and the modifications of oligonucleotides with indole-dU (W), phenol-dU (Y), and Amine-dU(X) were successful with the Cloud Intelligence Software of AM-Biotech (Fig. 2B).

**The test concentration of nSAG1 and affinity of the aptamers**

Since the aptamers were screened using the r-tSAG1 protein, to determine the efficacy of the r-tSAG1-recognized aptamers for the detection of the nSAG1, we measured the optimal concentration of WH3Ag containing the nSAG1 which could bind to the aptamers. The results demonstrated 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-tSAG1-positive control (Fig. 3). The affinity of four aptamer molecules to nSAG1 was then determined on the plate coated with WH3Ag (Fig. 4A). For affinity test, experimental absorbance and concentration data were used to calculate the dissociation constant(Kd). By using the nonlinear regression equation \( y = \frac{B_{\text{max}} \times x}{x + K_d} \), where B_{\text{max}} is the maximal affinity and Kd 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 Kd value. The Kd 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 feasible in DELAA for detection of nSAG1 in the subsequent measurements (p < 0.05) (Fig. 4B).

**Detection Of Nsag1 In Mouse Sera By Delaa**

In view of the highest avidity of apramer-2 to nSAG1, 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 normal sera significantly decreased when the sera were diluted to more than 1:10 (Fig. 5A), and the optimal dilution of HRP-avidin was 1:10 000 (Fig. 5B). 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 mice infected with WH3 strain parasite on different days of infection and from uninfected mice were collected and analyzed using DELAA. The results showed that mice presented positive nSAG1 on day 3 and gradually reached the highest OD value...
on day 7 after infection (Fig. 5C) \( p < 0.001 \). Expectedly, the OD values varied in mice infected with the low virulent and cyst-forming WH6 strain based on the parasite loads, reaching a high level from day 5 to 7, and remarkably decreased on day 14 postinfection (Fig. 5D) \( p < 0.001 \). However, no significant difference of OD450 values was noted with different doses via gavage on the same day of infection \( P > 0.05 \).

**The nSAG1 detection by DELAA in human sera of T.gondii infection**

Twenty human serum samples which were previously negative for circulating antigens tested by *Toxo*PCR test kit were examined to determine diagnostic threshold of DELAA. The cut-off value was found to be 0.46 (mean + 2SD). Finally, 35 negative and 15 positive sera which had been confirmed by the *Toxo*PCR test kit were re-tested with both DELAA and *Toxo*CAg-ELISA simultaneously. The results revealed that the sensitivity and specificity of DELAA and *Toxo*CAg-ELISA were 80% and 94.28%, and 66.60% and 82.85%, respectively (Table 1), suggesting a better efficacy of DELAA than *Toxo*CAg-ELISA in sensitivity \( \chi^2 = 5.14, P < 0.05 \) (Table 2) and specificity \( \chi^2 = 4.0, P < 0.05 \) (Table 3) in diagnosing acute *Toxoplasma* infection.

Table 1 A comparison of DELAA and *Toxo*CAg-ELISA using PCR-positive and negative human sera for diagnosis of *T.gondii* infection

| PCR | DELAA |  | ToxoCAg- ELISA |
|-----|-------|---|----------------|
|     | +     | - | +              |
|     | 12    | 3 | 10             |
|     | 2     | 33| 6              |
|     | 33    | 6 | 29             |

The sensitivity and specificity of DELAA and *Toxo*CAg-ELISA were 80% and 94.28%, and 66.60 % and 82.85%, respectively.

Table 2 A comparison of DELAA with *Toxo*CAg-ELISA using PCR-positive human sera for diagnosis of *T.gondii* infection

| DELAA | ToxoCAg- ELISA |
|-------|----------------|
| +     | 7              |
| -     | 7              |

\( \chi^2 = 5.143, P < 0.05 \)
Discussion

Toxoplasmosis is one of the zoonotic diseases which may deleteriously impact on three groups of individuals such as the pregnant women whose primary infection after gestation may cause adverse pregnant outcomes or congenital toxoplasmosis in the fetus[19], immunocomprised patients with reactivation of dormant infection, and immunocompetent individuals without distinct clinical manifestations. Lethal cerebral toxoplasmosis occurs in approximately 10–25% of *Toxoplasma*-seropositive patients, which is frequently caused by reactivation of latent infection of low virulent strains in immunodeficient patients, even leading to death[20]. Additionally, toxoplasmosis is also responsible for considerable economic losses of livestock production[21].

The conventional diagnosis includes detection of IgG and/or IgM, and IgA antibodies against *Toxoplasma* in sera of human or animals using a variety of methods. These antibody-based techniques 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)[22, 23]. Treatment is not usually recommended in the individuals of asymptomatic latent infection with only positive anti-*Toxol*gG antibodies.

On the other hand, a variety of molecules are actively secreted/excreted by *T. gondii* on the time of and during tachyzoite invasion of host cells and are an essential part of circulating antigens(CAs)[24]. 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 [25, 26]. These CAs/ESAs are a panel of good candidates for investigation into new diagnostic markers[27, 28], and detection of these antigenic substances using specific antibodies have been shown to be considerably useful[29]. Among them, surface antigen 1(SAG1), previously named as p30, is a predominant molecule on the membrane of tachyzoites[30]. SAG1 is a stage-specific antigen present only in tachyzoites with 336 amino acids [31] 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[12]. Having excellent immune features, recombinant SAG1 has been the most studied antigen of *Toxoplasma* for development of antibody-based serological tests and vaccine[32–34]. A previous study by Letillois and collaborators showed that rSAG-1 may be used as a marker of reactivated toxoplasmosis in HIV infected patients[11]. 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[11, 12].

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[35]. Aptamers provide a powerful tool not only used for identification of novel diagnostic markers but also 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[36–38].

In this study, we generated ssDNA aptamers against recombinant SAG1(r-tSAG1) of *Toxoplasma* by the SELEX technology (Fig. 2). With the screened and enriched 4 aptamers(Fig. 2), a direct enzyme-linked aptamer assay(DLAA) was primarily evaluated using r-tSAG1 and total lysates of *T. gondii* WH3 strain (WH3Ag), a virulent strain of type Chinese 1 dominating in animals and human of China(Fig. 3). Additionally, sera were collected from the suspected individuals with *Toxoplasma* infection confirmed by PCR examination, and those who were seropositive for anti-*Toxoplasma* IgM antibodies. The conditions such as the dilution of serum and streptavidin-HRP to be used in DELAA were optimized to provide the best test performance. We found that aptamer-2 was the appropriate detective probe for tests due to its high affinity to the native SAG1 protein (nSAG1) compared with the others (Fig. 4). We developed this novel aptamer-based method and used it for the detection of nSAG1 in the sera of animals and human with *Toxoplasma* infection. The detection limit of the DELAA with aptamer-2 was evaluated with nSAG1 diluted in mouse and human sera, respectively. The OD values of whole cell antigen of WH3 strain parasite, when diluted to the concentration of 1.56 (µg/ml), could be clearly differentiated from negative control of BSA when compared with positive control of purified r-tSAG1(Fig. 3). The mouse sera showed positive reaction on day 3 after virulent WH3 strain infection whereas the positive reaction was seen on day 7 following low-virulent WH6 strain infection (Fig. 5). Additionally, using an OD 0.47 as the cut-off value tested in 1:10 dilution with 20 normal human serum samples, we found that aptamer-2 could recognized 12 out of 15 sera of patients that positive for PCR test, with 80.00% sensitivity and 94.28% specificity. Comparatively, the commercial kit for detection of *Toxoplasma* circulating antigen by ELISA (*Toxo*Cag-ELISA) had a sensitivity of 66.6% and a specificity of 82.85% (Table 1), indicating that aptamer-2 based DELAA has a higher test performance compared to the antibody based *Toxo*Cag-ELISA for human diagnosis of toxoplasmosis (p<0.05)(Table 2,3). Our results suggest that aptamer-2 based technique may provide a novel strategy for detection of *T.gondii* SAG1 protein that could aid in the point-of-care diagnosis of acute or reactivated toxoplasmosis due to its low cost, high reliability, and rapid diagnostic procedures.
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 the 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, the 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 merely for major infectious diseases but also for cancers.

Aptamers-based technique has been employed in the detections of Mycobacterium, Salmonella, Listeria, Staphylococcus, Clostridium, Bacillus, and Escherichia, Aspergillus and Penicillium, and HBV, HCV, HIV, SARS-CoV, influenza virus, respiratory syncytial virus, and prion protein as well[39]. In the last decade, considerable achievements were made using aptamer-based assay in diagnosing some of the most challenging protozoan parasites infecting humans such as malaria, trypanosomiasis, leishmaniasis, amoebiasis, and cryptosporidiosis in laboratory settings[15]. Lou Y et al. selected two specific aptamers of TGA6 and TGA7 targeting anti-ToxoIgG and developed a quantum dots-labeled dual aptasensor (Q-DAS). The TGA6-anti-ToxoIgG-TGA7 sandwich method was used for detection of IgG antibodies against T.gondii, showing as high as 94.8% sensitivity and 95.7% specificity by reading the labeled fluorescence when compared with antibody-based immunoassay[16]. Recently, an enzyme-linked aptamer assay (ELAA) with new aptamers (AP001 and AP002) against ROP18 protein, a crucial virulent factor secreted by Toxoplasma, were established and used to evaluate total protein from T. gondii RH strain and recombinant ROP18[17]. The ELAA demonstrated higher affinity and specificity to RH strain antigen and rROP18. Detection limit of rROP18 in sera reached a low concentration of 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 but a decreased immunogenetic antigen of Toxoplasma tachyzoite[40], SAG1 is believed to be the most reliable marker targeted by its recognition aptamers.

In conclusion, we developed a direct enzyme-linked aptamer assay (DELAA) with aptamer-2 recognition probe for the detection of native SAG1 protein of T.gondii tachyzoite, which has high sensitivity, specificity and reproducibility. The novel aptamer-based technique is considered as a promising and efficient 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 during the current study are included in this published article and its additional files.

**Ethics approval and consent to participate**

Ethical permission was obtained from Institutional Review Board of AMU Institute of Biomedicine AMU (Permit No. AMU26–081108).

**Authors’ contributions**

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

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Figures**
Figure 1

Soluble expression and identification of Toxoplasma r-tSAG1. (A) insoluble expression of r-tSAG1 in sediments 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-tSAG1 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-tSAG1; 5 and 6, supernatants and sediments of the lysates following treatment of 0.01M Tris-Cl in PBS); (C) r-tSAG1 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-tSAG1 by nickel column); and (D) r-tSAG1 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-tSAG1 by nickel column).
Figure 2

Optimization of aptamers gene amplification by PCR. (A) Twenty-five cycles lead to abundant production of PCR; and (B) Four aptamers with given conformations were screened and identified by second-generation sequencing.
Figure 3

Definition of optimal dilution of the nSAG1 in WH3Ag when detected by DELAA. The WH3Ag was serially diluted from 100μg/mL to 1.56μg/mL. The r-tSAG1 and BSA at 1.56μg/mL were taken as positive and negative controls. It shows that a concentration of 1.56μg/mL WH3Ag gave rise to the best performance (*p<0.05; **p<0.01; ***p<0.001)
Figure 4

Affinity of the four aptamers binding to the nSAG1 in WH3Ag tested by DELAA. (A) A serial dilution of the aptamers from 200μg/mL to 1.56μg/mL was used, and BSA served as negative control; and (B) Nonlinear regression analysis of $y = (B_{max}x) / (x + K_d)$ indicates that aptamer-2 has a smallest Kd value (41.57±9.70), suggesting a highest avidity to nSAG1 compared with the other three aptamers (*p<0.05; **p<0.01; ***p<0.001).
Figure 5

Optimization of working dilution of mouse serum and HRP-avidin in DELAA for detection of nSAG1. (A) The ratio of OD value using pooled sera of Toxoplasma-infected mice to normal animals decreased notably when the dilution was more than 1:10; (B) HRP-avidin diluted at 1:10000 resulted in an optimal working concentration; Positive/Negative(P/N) was statistically significant under this condition. (C) Mice infected with virulent WH3 strain of T.gondii presented a strongly positive OD value (OD450nm: 0.63, cut-off value: 0.38) when detected at different time post-infection; (D) 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 post-infection with DELAA. (*p<0.05; **p<0.01; ***p<0.001).