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Prokaryotic Expression of the *Toxoplasma gondii SAG2* Gene in Pet Cats and Establishment of an Indirect ELISA Method

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LC designed the study and drafted the paper. LC and AL validated sequencing data by ELISA. YC and JZ conducted the research and analysed data. ZL contributed to the discussions and to draft the final version of the manuscript.

Key words
Indirect ELISA, Pet cats, Prokaryotic gene expression, *Toxoplasma gondii*, *SAG2* gene

ABSTRACT

This study aimed to establish an indirect enzyme-linked immunosorbent assay (ELISA) method based on the *SAG2* gene of *Toxoplasma gondii* (*T. gondii*) to improve the detection of toxoplasmosis in pet cats. *Escherichia coli* BL21 (DE3) was transformed with a prokaryotic expression vector, pET-21a-SAG2, which was constructed and induced to express a recombinant protein (SAG2), identified using SDS-PAGE and Western blot analysis. The purified protein was then used as a coating antigen to establish an indirect ELISA method for detecting the *T. gondii* antibody in pet cats, whose reaction conditions were optimized. The *SAG2* gene was successfully cloned into a pET-21a (+) prokaryotic expression vector, and the recombinant plasmid pET-21a-SAG2 was obtained. The size of the recombinant protein was approximately 19 kDa, and it was expressed mainly in the form of an inclusion body. The optimal reaction conditions were as follows: antigen-coating concentration, 0.6 $\mu g \cdot mL^{-1}$; serum dilution, 1:200; 60-min serum action time, 1:2000 working dilution of the enzyme-labeled secondary antibody; and 5% skimmed milk used as a blocking solution. No cross-reactivity was observed with the positive serum of *Eperythrozoon*, *Trichinella spiralis (T. spiralis)* and *Hydatid cysts*. The coefficients of the inter- and intraassay variations in the repeatability tests were lower than 10%. The established method and the indirect hemagglutination (IHA) test were used to detect 50 clinical samples at the same time. The positive coincidence rate of the two was 94.11%. Therefore, the newly established indirect ELISA method had high sensitivity, specificity, stability, and intra- and interbatch repeatability. It could be used for the diagnosis and epidemiological investigation of toxoplasmosis in cats, thus laying a good foundation for preparing ELISA kits for clinical detection.

INTRODUCTION

Toxoplasmosis, caused by *Toxoplasma gondii (T. gondii)*, is a worldwide distributed zoonotic parasitic disease that seriously endangers human health and animal husbandry development. *T. gondii*, which is characterized by bow-shaped trophozoites (Wang *et al*., 2008; Montoya, 2002), can infect humans and almost all warm-blooded animals (Mohamed, 2020). It is manifested as a recessive infection in adults and causes abortion, stillbirth, or fetus weakness in pregnant women (Slavin *et al*., 1994; Xiao *et al*., 2010). This zoonotic pathogen can also infect pregnant animals, causing serious damage to their production and serious economic losses to animal husbandry (Li *et al*., 2014). As the only terminal hosts of *T. gondii*, the Felidae family representatives are among the main toxoplasmosis infection sources (Wu *et al*., 2011), and hence of substantial public health significance. The number of pet cats has steadily increased with the rapid development of China's economy and the improvement in living standards, increasing the contact opportunities between people and cats and thus also the probability of *T. gondii* infection. Therefore, the importance of pet cats for the spread of *T. gondii* infection should not be ignored (Tenter *et al*., 2000; Hill and Dubey, 2002). Since most cats show subclinical symptoms after *T. gondii* infection (Duan *et al*., 2012; Cui *et al*., 2012), serological and molecular biological detection has become the main approach for the

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research of toxoplasmosis prevalence in cats.

The clinical manifestations of toxoplasmosis are diverse and complex. Also, the clinical diagnosis is difficult to make, thus increasing the reliance on laboratory diagnostic methods. Extensive research has been conducted at home and abroad on the methods for diagnosing *T. gondii*. Currently, the most commonly used *T. gondii* diagnostic methods are etiological (Cao *et al*., 2015b), molecular biological (Wang *et al*., 2017), and immunological (Hajissa *et al*., 2018). However, the etiological examination is not suitable for grassroots applications due to its low detection rate, complex technology requirements, cumbersome operation, expensive equipment, and so forth (Vazini *et al*., 2018). Polymerase chain reaction (PCR) and DNA technologies in molecular biological diagnostic methods need to be simplified and improved due to their demanding professional requirements for operation and high cost (Liu, 2017). The immunological diagnosis by enzyme-linked immunosorbent assay (ELISA) is feasible because it is relatively sensitive, specific, and simple, and results can be observed with naked eyes. Thus, it has become one of the most widely used techniques for diagnosing toxoplasmosis (Song *et al*., 2016; Gong *et al*., 2017).

Recombinant surface antigen 2 (*SAG2*) is a surface antigen protein with strong antigenicity and immunogenicity (Zhang, 2019; Tao, 2010). *SAG2* is expressed only in the tachyzoites in the invasive stage, but not in the sporozoite and bradyzoite stages; it has a strong immune-induction effect. The reaction intensity of recombinant *SAG2* antigen in detecting acute infection serum is significantly higher than that in detecting chronic infection serum, and hence it can be used for diagnosing acute *T. gondii* (Quan *et al*., 2014). In a previous study, the soluble SAG2 protein of *T. gondii* was overexpressed in *Escherichia coli*, inducing a significantly higher serum reaction rate in patients with *T. gondii* infection in the acute phase than in patients with chronic infection, confirming the natural immune activity of the recombinant protein (Lau and Fong, 2008).

In this study, the *T. gondii SAG2* gene was cloned and prokaryotically expressed. Then, the SAG2 prokaryotic expression recombinant protein was used as a coating antigen to establish an indirect ELISA method with high sensitivity, strong specificity, and good repeatability for detecting the *T. gondii* antibody in pet cats.

MATERIALS AND METHODS

Insect species and serum

The pM19-T-SAG2 plasmid and *Toxoplasma* HBBD2013 strain were preserved in the Parasite Laboratory of the School of Animal Medicine, Hebei Agricultural University (Baoding, Hebei, China). The positive and negative sera of *T. gondii*, *Eperythrozoon*, *Trichinella spiralis (T. spiralis)*, and hydatid cysts were also stored in the Parasite Laboratory of the School of Animal Medicine, Hebei Agricultural University.

Clinical sample collection

A total of 50 blood samples of pet cats were collected from 10 pet hospitals in Baoding, Hebei Province; the serum was separated from the samples and stored at −80°C for later use.

Main reagents

Plasmid DNA extraction kit, trans5 α-competent cells, and pET-21a (+) plasmid were purchased from TaKaRa Company (Dalian, China). DNA recovery kit and 3,3-diaminobenzidine (DAB) chromogenic solution were purchased from Beijing Solebao Technology Co., Ltd. (Beijing, China). Ni-agarose resin were bought from Beijing Kangwei Century Biotechnology Co., Ltd. (Beijing, China). *Bam*HI and *Xho*I were purchased from New England Biolabs Co., Ltd. (USA). *E. coli* BL21 (DE3)-infected cells were obtained from Kangwei Century Biotechnology Co., Ltd. (Beijing, China). The HRP-labeled goat anti-cat secondary antibody was purchased from Jackson Immune Research Co., Ltd. (PA, USA).

Design and synthesis of primers

A pair of *SAG2* genes of *T. gondii* were designed based on an NCBI GenBank gene sequence (accession no. FJ825705) using Primer 5.0 software. The expected amplified gene fragment had a length of 570 bp. *Bam*HI and *Xho*I restriction sites and protective bases were added to the upstream and downstream 5’ ends, respectively. The primers were synthesized by Beijing Nuosai Gene Biotechnology Co., Ltd. (Beijing, China). The sequences of primers were as follows:

F: 5′-ATTGGATCCATGCTTGCGTCCACCACCG-3′
R: 5′- CACCTCGAGTTACTTGCCCGTGAGA -3′

SAG2 *gene cloning and recombinant plasmid construction*

The pM19-T-SAG2 plasmid was used as a template for PCR amplification. A volume of 50 μL of the PCR reaction system contained the following reagents: 2 μL of pM19-T-SAG2 plasmid, 1 μL of upstream and downstream primers (10pM), 10 μL of 5× Phusion High Fidelity Enzyme PCR buffer, 1.25 μL of 10mM dNTP, and 1 μL of Pfu high-fidelity enzyme; DEPC water was added to this mixture to reach 50 μL of the reaction system. The following PCR reaction conditions were employed: pre-denaturation at 98°C for 5 min; denaturation at 98°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, a total number of 30 cycles, followed by storage

at 4°C. The obtained PCR products were identified by 1% agarose electrophoresis, and the *SAG2* gene fragment was purified using a gel recovery kit (Tiangen, Beijing, China). The *SAG2* gene fragment was digested with restriction endonucleases *Bam*HI and *Xho*I, and the prokaryotic expression vector pET-21a (+) was ligated overnight with T4 ligase at 16°C. The recombinant plasmid was then transformed into *E. coli* trans5 α-competent cells. The expression plasmid pET-21a-SAG2 was identified by PCR, double-digestion, and sequencing.

Induction and expression of recombinant protein

The identified correct recombinant plasmid pET-21a-SAG2 was transformed into *E. coli* BL21 competent cells. Next, a single colony was selected, inoculated into Amp-containing LB medium, and cultured with shaking at 37°C overnight. Then, 1:100-diluted fresh LB medium was added, and the culture at 37°C was continued. When the bacterial solution OD_{600} = 0.4–0.6, 1 mmol·L^{-1} IPTG was added to induce expression at 37°C. After 8 h of expression, the cells were collected, ultrasonically lysed, and centrifuged, and the supernatant and precipitate were subjected to SDS-PAGE for determining their enlargement and solubility.

Purification and Western blot analysis of the recombinant protein

After ultrasonic crushing, the inclusion bodies were centrifuged, precipitated twice with an appropriate amount of washing solution (50mM Tris-HCl, 10mM EDTA, 0.5% Triton X-100, and 0.15 M NaCl), and then centrifuged again to obtain preliminary purified inclusion bodies.

The inclusion bodies were next added to a ninefold volume of inclusion body lysate (50mM Tris-HCl, 2mM EDTA, 6M guanidine hydrochloride, and 10mM DTT). After violent vibration, the inclusion bodies were completely dissolved at 4°C overnight. After centrifugation, the protein in the supernatant was denatured and transferred onto a balanced Ni-affinity chromatography column. Further, the target protein was eluted with 500mM imidazole, the eluate was collected, and the inclusion body refolding solution (50mM Tris-HCl, 0.2mM EDTA, 0.5M L-Arg, 1mM GSSG, 3mM GSH, 1M guanidine hydrochloride, and 0.15 M NaCl) was added. The mixture was left undisturbed for 4 h at 4°C and then centrifuged. The supernatant was collected to obtain the refolded recombinant protein. The solution was placed in a dialysis bag and dialyzed with 0.01M PBS (pH 7.2) at 4°C for 72 h. After aseptic filtration, the purified recombinant protein was measured by Nanodrop 2000 UV spectrophotometer, and analyzed using SDS-PAGE.

Next, the recombinant protein was transferred onto the nitrocellulose membrane and then blocked overnight with PBST containing 5% BSA. The mouse anti-*T. gondii* antibody was used as the primary antibody, and rabbit anti-mouse IgG labeled with HRP was used as the secondary antibody. The recombinant protein was incubated at 37°C for 2 h and stained with a DAB color reagent kit.

Establishment of indirect ELISA

Determination of the optimal concentration of antigen coating and serum dilution

The optimal reaction conditions were determined by square titration. The purified recombinant SAG2 protein solution was diluted with 4.8 μL·mL^{-1} of 0.05M, pH 9.6, carbonate buffer solution. Each well of the enzyme plate was coated with 100 μL of diluted recombinant SAG2 protein solution and incubated overnight at 4°C. The positive and negative sera were diluted from 1:50 to 1:2000. The remaining steps were carried out following the manufacturer's protocols for ELISA. Finally, 100 μL/well of TMB substrate was added, and the reaction was terminated by adding 100 μL/well of 2M concentrated sulfuric acid for 5–10 min. Further, the $OD_{450\ nm}$ value was measured using ELISA. The OD values of the positive and negative sera were compared, and the ratio of the OD value (P/N value) of the corresponding positive serum (P) to that of the negative serum (n) was calculated. The OD_{450nm} value of the positive serum was close to 1.0. The antigen-coating concentration and the serum dilution degree corresponding to the hole with the largest P/N value were considered the optimal antigen-coating concentration and serum dilution degree.

Determination of the best working concentration of enzyme-labeled secondary antibody

Using the optimal antigen-coating concentration and serum dilution, the goat anti-cat IgG HRP secondary antibody was diluted with 1:1000–1:4000 for indirect ELISA determination to establish the optimal working concentration of the enzyme-labeled secondary antibody.

Selection of the best sealing fluid

The optimal antigen-coating concentration, serum dilution, and working concentration of the secondary antibody were further used. Next, 1% alum, 1% BSA, 5% bovine serum, and 5% skimmed milk were selected as the blocking liquids to determine the optimal sealing liquid.

Determination of the optimal working concentration of the serum

The positive and negative serum were treated with the optimal antigen-coating concentration, blocking solution, serum dilution, and the best secondary antibody

concentration for 30, 60, 90, and 120 min, respectively. The OD_{450nm} value was then measured to determine the optimal working time of the first antiserum.

Optimal working duration of the enzyme-labeled secondary antibody

The enzyme-labeled secondary antibody was treated with the best antigen-coating concentration, blocking solution, serum dilution, and the optimal dilution concentration of the secondary antibody for 30, 60, 90, and 120 min, respectively. The $OD_{450\ nm}$ value was measured to determine the optimal working time of the enzyme-labeled secondary antibody.

Determination of the critical value of the negative and positive indirect ELISA

A total of 40 cat-negative sera stored in the laboratory (determined as negative using a commercial ELISA kit) were tested using the established indirect ELISA method. Then, the average (X(—)X) and standard deviation (SD) of the sample OD_{450nm} value were calculated. According to statistical principles, when the OD_{450nm} value of the sample was X(—)X + 3 SD of the OD_{450nm} value of the negative sample, it was judged as positive at the level of 99.9%.

Specificity test

The positive sera of *Eperythrozoon*, *T. spiralis,* and *Hydatid cysts* were analyzed using the newly established indirect ELISA method to determine whether they had cross-reactivity with the positive sera of other parasites.

Sensitivity test

The standard positive serum of *T. gondii* was diluted 1:200–1:25,600 times. The optimal reaction conditions were applied.

Repeatability test

The new indirect ELISA method was used to determine five positive sera with different antibody titers (commercially available ELISA kits were used to determine the positive sera). The serum was diluted 1:200, and each serum sample was assessed in three wells in parallel to calculate the intra-assay coefficient of variation. Then, three plates were subjected to repeated tests to calculate the coefficient of variation between batches.

Clinical sample testing

According to the established ELISA method and indirect hemagglutination (IHA) test, 50 serum samples were detected at the same time. The positive detection rate and coincidence rate of the two methods were compared.

RESULTS

Expression of SAG2 *gene of* T. gondii

The pM19-T-SAG2 plasmid and the designed primers were used as a template for PCR amplification. After electrophoresis of the PCR product, a 570-bp band was obtained, as expected (Fig. 1).

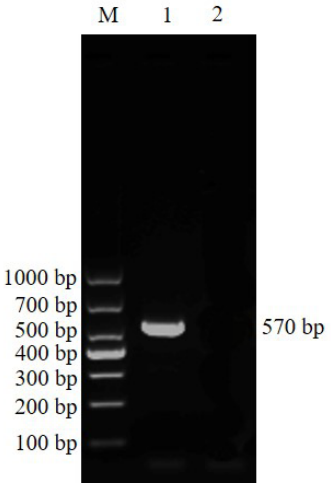

Fig. 1. Amplification of the *SAG2* gene of *T. gondii.* M, DL1000 DNA marker; 1, *SAG2* gene of *T. gondii*; 2, negative control results.

The *SAG2* gene fragment was ligated to the pET-21a prokaryotic expression plasmid. After the recombinant expression plasmid was digested with endonucleases *Bam*HI and *Xho*I, a 570-bp target fragment and pET-21a vector fragment were obtained, as shown in Figure 2. The results of plasmid sequencing showed that the recombinant plasmid pET-21a-SAG2 was constructed correctly.

SDS-PAGE results showed that pET-21a-SAG2, which was transformed into *E. coli* BL21 (DE3) competent cells and induced by ITPG, was expressed as a fusion protein with a molecular mass of approximately 19 kDa, which was in line with the expected size. The recombinant bacteria were lysed by ultrasound, and the supernatant and the precipitate were collected for electrophoresis. The target protein was present mostly in the precipitate after bacterial lysis, as depicted in Figure 3, indicating that the recombinant protein existed mainly in the form of inclusion bodies.

SAG2 protein

The denatured protein purified by Ni agarose was renatured in a refolding solution containing 1M guanidine hydrochloride. The recombinant protein was analyzed by SDS-PAGE. The results showed that the purity of the

refolded recombinant protein was higher than 95%, and the purified protein had a single band with good purification effect, as shown in Figure 4.

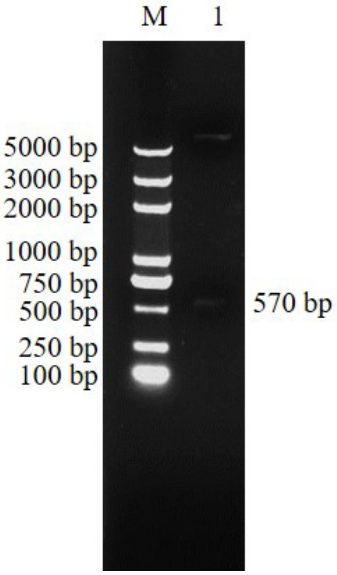

Fig. 2. Restriction enzyme digestion analysis results of pET32a-SAG2. M, DL2000 plus DNA marker, 1, The product of recombinant plasmid double-enzyme digestion.

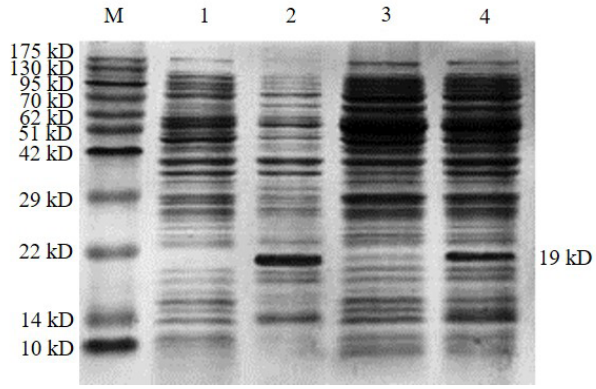

Fig. 3. Expression of *SAG2* gene in *E. coli* BL21. M, Marker; 1, Control plasmid expression; 2, Precipitation of recombinant bacteria; 3, Supernatant recombinant bacteria; 4, Recombinant bacteria.

Western blot analysis results showed that the purified protein reacted with the positive serum of *T. gondii* (Fig. 5), indicating that the recombinant protein was correctly expressed in *E. coli* and had good immunogenicity.

The purified recombinant protein was measured by a Nanodrop 2000 UV spectrophotometer, and the protein concentration was found to be 0.4806 mg/mL.

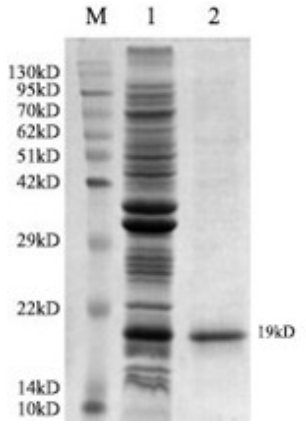

Fig. 4. Recombinant *SAG2* protein purification. M, Marker: (1) Precipitation of recombinant bacteria induced; (2) Purified recombinant protein.

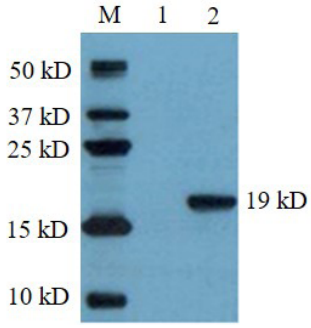

Fig. 5. Recombinant protein identification by Western blot. M, Marker; (1) blank control; (2) purified recombinant protein.

Optimum conditions for indirect ELISA method

For optimal antigen-coating concentration and serum dilution Table I shows the square matrix titration results. All P/N values were the highest at an antigen-coating concentration of 1.2 μg·mL^{-1} or 0.6 μg·mL^{-1} and a serum dilution of 1:200 (15.52 and 13.98, respectively). However, the OD_{450nm} value of the positive serum was closer to 1.0 at an antigen-coating concentration of 0.6 μg·mL^{-1}. Therefore, the optimal coating concentration of the antigen was 0.6 μg·mL^{-1}, and the best serum dilution was 1:200.

Table II shows the best working concentration of enzyme-labeled secondary antibody. The results showed that the OD_{450nm} value of the positive serum was approximately 1.0, the negative value was less than 0.2, and the P/N value was the highest at a working concentration of the enzyme-labeled antibody of 1:2000. Therefore, the optimal working concentration of the enzyme-labeled antibody was 1:2000.

Table I. Determination of the optimal coating concentration and serum dilution of the antigen by square titration.

	Serum dilution	Coating concentration (μg·mL^{-1})					
		4.8	2.4	1.2	0.6	0.3	0.15
Positive serum	1:50	2.258	2.239	2.186	1.949	1.002	0.621
	1:100	1.971	1.957	1.957	1.572	0.576	0.479
	1:200	1.727	1.671	1.568	1.273	0.464	0.382
	1:400	1.483	1.474	1.436	0.948	0.339	0.223
	1:800	1.103	0.997	0.744	0.739	0.391	0.162
Negative serum	1:50	0.212	0.215	0.191	0.198	0.117	0.086
	1:100	0.187	0.187	0.153	0.121	0.097	0.072
	1:200	0.151	0.129	0.101	0.091	0.074	0.064
	1:400	0.108	0.115	0.095	0.078	0.060	0.054
	1:800	0.066	0.069	0.068	0.058	0.065	0.078

Table III shows that the highest P/N value was obtained when 5% skimmed milk was used as the sealing liquid, and it was thus selected for use in further experiments.

Table IV shows that the highest P/N value was obtained when the serum action time was 60 min.

Table V shows that the P/N value was the highest when the secondary antibody action time was 30 min.

Forty cat serum samples previously found to be negative using commercial ELISA kits were tested by the newly established ELISA method. The final average OD_{450nm} value of the 40 negative sera was calculated to be 0.184, with an SD of 0.050, which was established in both the negative and positive controls. When the final value of OD_{450nm} was ≥0.334, it was judged to be positive for the anti-*T. gondii* antibody, and when the final value of OD_{450nm} was less than 0.334, it was considered to be negative for the anti-*T. gondii* antibody. The OD_{450nm} values of the 40 serum samples are listed in Table VI.

Table II. Determination of the concentration of enzyme-labeled antibody.

	Dilution of enzyme-labeled secondary antibody											
	1:1000			1:2000			1:3000			1:4000		
Positive serum	2.029	1.952	1.929	1.226	1.266	1.213	0.979	0.853	0.842	0.765	0.769	0.758
Negative serum	0.204	0.176	0.188	0.093	0.083	0.091	0.085	0.065	0.073	0.088	0.072	0.072
P/N value	10.41			13.88			11.99			9.88		

Table III. Determination of the best blocking solution.

	1% alum			1%BSA			5% Bovine serum			5% skimmed milk		
Positive serum	1.341	1.247	1.163	1.230	1.280	1.217	1.241	1.144	1.184	1.186	1.173	1.108
Negative serum	0.177	0.171	0.191	0.183	0.177	0.173	0.155	0.138	0.168	0.059	0.066	0.069
P/N value	6.96			6.99			7.74			17.87		

Table IV. Determination of the best duration of serum action.

	Serum action time (min)											
	30			60			90			120		
Positive serum	1.107	1.127	1.099	1.213	1.141	1.141	1.231	1.278	1.197	1.447	1.501	1.499
Negative serum	0.097	0.082	0.086	0.075	0.090	0.074	0.096	0.123	0.119	0.193	0.13	0.139
P/N value	12.58			14.62			10.96			9.63		

Table V. Determination of the optimal working duration of the enzyme-labeled secondary antibody.

	Time of second antiviral action (min)											
	30			60			90			120		
Positive serum	1.011	1.171	1.188	1.352	1.366	1.323	1.380	1.501	1.419	1.485	1.397	1.502
Negative serum	0.103	0.111	0.109	0.215	0.193	0.205	0.244	0.203	0.247	0.295	0.266	0.293
P/N value	10.43			7.15			6.20			4.73		

Table VI. $OD_{450\ nm}$ values of 40 negative serum samples.

Negative serum OD_{450nm}				
0.182	0.243	0.114	0.156	0.170
0.219	0.127	0.243	0.162	0.162
0.172	0.260	0.139	0.172	0.206
0.230	0.237	0.169	0.184	0.172
0.293	0.124	0.135	0.130	0.187
0.288	0.121	0.170	0.122	0.289
0.129	0.159	0.188	0.186	0.213
0.167	0.234	0.190	0.207	0.109

Specificity of ELISA method

The $OD_{450\ nm}$ value of *Eperythrozoon*, *T. spiralis*, and hydatid cysts was 0.217, 0.138, and 0.156, respectively, which were all lower than the critical value of 0.334, indicating that the indirect ELISA method had good specificity.

Sensitivity of ELISA method

The antigen was coated using the optimal concentration and other conditions. Then, *T. gondii* positive serum was diluted 1:200–1: 25,600 times, and the other optimal reaction conditions were used for indirect ELISA. As shown in Table VII, 1:3200 dilutions were still positive, but the detection result of 1:6400 dilution of the positive serum was lower than 0.334, indicating that the novel indirect ELISA had good sensitivity.

Table VII. Sensitivity test results.

Serum dilution	Positive serum			Mean	Negative serum			Mean
1:200	2.358	2.251	2.223	2.277	0.112	0.133	0.164	0.136
1:400	1.914	1.623	1.604	1.714	0.118	0.098	0.106	0.107
1:800	1.174	1.068	1.051	1.098	0.068	0.068	0.060	0.065
1:1600	0.684	0.622	0.612	0.639	0.056	0.055	0.053	0.055
1:3200	0.399	0.378	0.365	0.381	0.056	0.053	0.050	0.053
1:6400	0.241	0.216	0.211	0.223	0.060	0.059	0.052	0.057
1:12,800	0.154	0.136	0.139	0.143	0.059	0.061	0.055	0.058
1:25,600	0.114	0.104	0.104	0.107	0.068	0.086	0.057	0.070

Repeatability of ELISA method

Five *T. gondii* serum samples with different antibody titers stored in the laboratory were detected three times with the same batch of coated enzyme plates,as shown in Table VIII. The intra-assay coefficient of variation (CV) was 2.9%–6.3%, and the coefficient of variation CV between batches was 5.3%–5.6% for the three plates, indicating that the intra- and interbatch variations of this method were very low and it thus had good repeatability.

Clinical sample with ELISA and IHA

The positive rate results of ELISA and IHA were compared thrice, as shown in Table IX. The ELISA method was used to detect antibodies in 50 serum samples, and the positive rate was 34%; The IHA method was used to detect the same 50 serum samples, and the positive rate was 32%. The positive coincidence rate of the two analyses was 94.11%, the negative coincidence rate was 100%, and the total coincidence rate was 98%.

Table VIII. Repeatability test results.

Sample No.	Intra assay variance		Difference between batches	
	OD_{450nm}	Coefficient of variation (%)	OD_{450nm}	Coefficient of variation (%)
1	1.125 ± 0.033	2.9	1.129±0.054	3.5
2	1.208 ± 0.044	3.6	1.211±0.065	5.3
3	0.673 ± 0.031	4.6	0.670±0.034	5.1
4	0.284 ± 0.016	5.6	0.290±0.016	5.6
5	0.431 ± 0.027	6.3	0.429±0.023	5.4

Table IX. Comparison of IHA and ELISA methods.

	Positive samples	Negative sample	Total	Positive rate (%)
ELISA	17	33	50	34
IHA	16	34	50	32

DISCUSSION

Currently, serological assessment is the main method for detectig *T. gondii* infection, including IHA test, indirect immunofluorescence, ELISA, and so forth (Apsari *et al*., 2012; Chen *et al*., 2013). The rapid development of commercial kits has increased the convenience of diagnosing infection caused by this pathogen. However, it is difficult for laboratory personnel to complete the analysis of results due to the large differences in detection ability, stability, and specificity, which affects the authenticity of the results (Niu *et al*., 2000; He *et al*., 2008). The ELISA method has many advantages, such as simple operation, simultaneous processing of a large number of samples, high specificity and sensitivity, and so forth, which has made it more suitable for clinical large-sample detection and screening (Lau and Fong, 2008). The operation of the instrument used in ELISA is relatively simple. Moreover,

ELISA results obtained by reading the absorbance of the enzyme plate and excluding the influence of subjective factors are more accurate than those obtained through IHA; hence, the former method is among the main approaches used for diagnosing *T. gondii* infection.

Domestic and foreign in-depth research has been carried out to detect the *T. gondii* antibody by ELISA. Common coating antigens employed in the ELISA detection of *T. gondii* are SAG1 (P30), SAG2 (P22), p35, and other proteins (Zhou *et al*., 2019; Chu *et al*., 2018; Cao *et al*., 2015a). Among these, the SAG1 protein is related to the infection caused by *T. gondii* and is the main target antigen. SAG1 of *T. gondii* has been previously cloned and expressed. Further research established that the SAG1 recombinant protein could be recognized by the positive serum of *T. gondii* through a strong specific reaction, and hence could be used for preparing a diagnostic test kit (Qi *et al*., 2020; Zhao *et al*., 2017; Wang *et al*., 2015).

The length of the SAG2 protein sequence was found to be 1404 bp, and the open reading frame encoded 597 amino acids. In a previous study, the recombinant SAG2 protein was used to immunize mice and evidenced to induce the production of mouse anti-SAG2 positive serum and activate cellular immunity, thus achieving certain immune protection in mice (Chen *et al*., 2013). Additionally, (Lau and Fong, 2008; Niu *et al*., 2000) expressed the *T. gondii* SAG2 protein in a eukaryotic yeast system, and the Western blot analysis showed that the recombinant protein specifically reacted with the positive serum. In this study, the SAG2 protein expressed in prokaryotic cells also had strong reactivity. Western blot analysis results confirmed that the recombinant SAG2 protein could be used as a coating antigen for establishing the indirect ELISA antibody method for detecting *T. gondii*.

Briefly, in the present study, the truncated *SAG2* gene was cloned and connected to a pET21a (+) vector, and a prokaryotic expression recombinant plasmid pET21a-SAG2 was constructed. After the recombinant expression plasmid was identified to have been correctly designed by double-enzyme digestion, it was transformed into the high-efficiency expression vector *E. coli* BL21 (DE3). The recombinant protein was expressed in the form of an inclusion body characterized by high protein concentration, easy purification, and high concentration after purification; hence it was suitable for preparing a detection antigen. Then, an Ni-affinity chromatography column was used to purify the recombinant protein, with a purification effect higher than 95%, which met the requirements of the follow-up tests. The recombinant SAG2 protein of *T. gondii* prepared in this study was stably expressed as a coating antigen. An indirect ELISA method was established for detecting the *T. gondii* antibody and the optimal reaction conditions were as follows: antigen-coating concentration, 0.6, established by square titration, $\mu g \cdot mL^{-1}$; serum dilution, 1:200; and reaction time, 60 min. The best working dilution of the enzyme-labeled secondary antibody was 1:2000, the best action time was 30 min, and 5% skimmed milk was the best blocking liquid; no cross-reactivity was observed with the positive serum of *Eperythrozoon*, *T. spiralis*, and *Hydatid cysts*. The coefficients of variation of inter- and intra-assay repeatability were lower than 10%. The established method and the IHA method were used to detect 50 clinical samples at the same time. The positive coincidence rate of the two was 94.11%.

Therefore, the indirect ELISA method established in this study had high sensitivity, strong specificity, and good stability. This novel technique was simple and easy for operation. The findings provided novel insights and opportunities for epidemiological investigation and control and prevention of toxoplasmosis in pet cats.

Ethical approval

All animal studies have been reviewed by the appropriate ethics committees.

Statement of conflict of interest

The authors have declared no conflict of interest.

REFERENCES

Apsari, I., Artama, W. and Damriyasa, I., 2012. Molecular diagnosis of *Toxoplasma gondii* based on the tachyzoite and bradyzoite stage specific genes in free-range chicken. *J. Vet*., **13**: 14-19.

Cao, A., Liu, Y., Wang, J., Li, X., Wang, S., Zhao, Q., Cong, H., He, S. and Zhou, H., 2015a. *Toxoplasma gondii*: Vaccination with a DNA vaccine encoding T- and B-cell epitopes of SAG1, GRA2, GRA7 and ROP16 elicits protection against acute toxoplasmosis in mice. *Vaccine*, **33**: 6757-6762. https://doi.org/10.1016/j.vaccine.2015.10.077

Cao, X., Xiong, S. and Yao, Z. 2015b. *Medical immunology*. People's Health Publishing House, Beijing

Chen, J., Huang, S.-Y., Li, Z.-Y., Yuan, Z.-G., Zhou, D.-H., Petersen, E., Zhang, N.-Z. and Zhu, X.-Q., 2013. Protective immunity induced by a DNA vaccine expressing eIF4A of *Toxoplasma gondii* against acute toxoplasmosis in mice. *Vaccine*, **31**: 1734-1739. https://doi.org/10.1016/j.vaccine.2013.01.027

Chu, J.-Q., Huang, S., Ye, W., Fan, X.-Y., Huang, R., Ye, S.-C., Yu, C.-Y., Wu, W.-Y., Zhou, Y., Zhou, W., Lee, Y.-H. and Quan, J.-H., 2018. Evaluation

of protective immune response induced by a DNA vaccine encoding GRA8 against acute toxoplasmosis in a murine model. *Korean J. Parasitol*., **56**: 325-334. https://doi.org/10.3347/kjp.2018.56.4.325

Cui, L., Yu, Y. and Liu, S., 2012. Epidemiological investigation of toxoplasmosis in dogs and cats in Beijing. *Chinese J. Vet. Med*., **48**: 7-10.

Duan, G., Tian, Y.-M., Li, B.-F., Yang, J.-F., Liu, Z.-L., Yuan, F.-Z., Zhu, X.-Q. and Zou, F.-C., 2012. Seroprevalence of *Toxoplasma gondii* infection in pet dogs in Kunming, Southwest China. *Parasit. Vectors*, **5**: 1-4. https://doi.org/10.1186/1756-3305-5-118

Gong, F., Jiang, W. and Chen, Y., 2017. Establishment of double antibody sandwich ABC-ELISA for detection of circulating antigen of *Toxoplasma gondii*. *Anim. Husb. Vet*., **49**: 65-70.

Hajissa, K., Zakaria, R., Suppian, R. and Mohamed, Z., 2018. Immunogenicity of multiepitope vaccine candidate against *Toxoplasma gondii* infection in BALB/c mice. *Iran. J. Parasitol*., **13**: 215.

He, Y., Jiang, S. and Ma, X., 2008. Evaluation of 16 diagnostic kits for *Toxoplasma gondii*. *J. Travel Med*., **14**: 43-45.

Hill, D. and Dubey, J.P., 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin. Microbiol. Infect*., **8**: 634-640. https://doi.org/10.1046/j.1469-0691.2002.00485.x

Lau, Y.L. and Fong, M.Y., 2008. *Toxoplasma gondii*: Serological characterization and immunogenicity of recombinant surface antigen 2 (SAG2) expressed in the yeast *Pichia pastoris*. *Exp. Parasitol*., **119**: 373-378. https://doi.org/10.1016/j.exppara.2008.03.016

Li, X.-L., Wei, H.-X., Zhang, H., Peng, H.-J. and Lindsay, D.S., 2014. A meta analysis on risks of adverse pregnancy outcomes in *Toxoplasma gondii* infection. *PLoS One*, **9**: e97775. https://doi.org/10.1371/journal.pone.0097775

Liu, W., 2017. *Establishment and preliminary application of ELISA and colloidal gold test paper for* Toxoplasma feline *infection*. Jilin University.

Mohamed, K., 2020. Toxoplasmosis in humans and animals in Saudi Arabia: A systematic review. *J. Infect. Dev. Count*., **14**: 800-811. https://doi.org/10.3855/jidc.12648

Montoya, J.G., 2002. Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. *J. Infect. Dis*., **185**(**s1**): S73-S82. https://doi.org/10.1086/338827

Niu, A., Feng, Y. and Liu, W., 2000. Comparison of preliminary detection results of several *Toxoplasma gondii* ELISA test kits. *Chinese J. Zoon*., **16**: 56-58.

Qi, T., Shi, C., Wang, Z., Zong, Y. and Li, J., 2020. Construction and expression of eukaryotic expression recombinant plasmid of sag1-gra1 complex gene of *Toxoplasma gondii*. *J. Guizhou med. Univ*., **45**: 286-291.

Quan, J.H., Li, P. and Yu, C., 2014. Immune protection of *SAG2* and gra2 gene vaccines against *Toxoplasma gondii*. *Acta Parasit. med. Entomol*., **21**: 227-232.

Slavin, M., Meyers, J., Remington, J. and Hackman, R., 1994. *Toxoplasma gondii* infection in marrow transplant recipients: A 20 year experience. *Bone Marrow Transpl*., **13**: 549-557.

Song, H., Yang, S., Gao, J., Gong, P., Li, J. and Zhang, X., 2016. Establishment of an indirect ELISA for detection of *Toxoplasma canis* SAG3 antibody. *Chinese J. Pathog. Boil*., **11**: 338-341, 345.

Tao, Y., 2010. Cloning, expression and preliminary identification of *SAG2* gene of *Toxoplasma gondii* Yangzhou University.

Tenter, A.M., Heckeroth, A.R. and Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol*., **30**: 1217-1258. https://doi.org/10.1016/S0020-7519(00)00124-7

Vazini, H., Ghafarifar, F., Sharifi, Z. and Dalimi, A., 2018. Evaluation of immune responses induced by *GRA7* and *ROP2* genes by DNA vaccine cocktails against acute toxoplasmosis in BALB/c mice. *Avicenna J. med. Biotechnol*., **10**: 2.

Wang, W., Feng, F., Lv, J., Xie, Z., Chen, J., Zhang, L. and Li, W., 2017. Major immunodominant region of hepatitis B virus core antigen as a delivery vector to improve the immunogenicity of the fusion antigen ROP2-SAG1 multiepitope from *Toxoplasma gondii* in mice. *Viral Immunol*., **30**: 508-515. https://doi.org/10.1089/vim.2016.0135

Wang, Y., Cao, J. and Sun, Y., 2015. Study on expression and immunogenicity of *Toxoplasma gondii* sagl, mic3, rop2 cocktail DNA vaccine with fluorescent protein reporter vector. *Chinese J. Parasitol. Parasitol*., **33**: 368-371-376.

Wang, Y., Li, X. and Zhang, D., 2008. Advances in diagnostic methods of toxoplasmosis. *Acta Zooepid. Sin*., **16**: 28-33.

Wu, S.-M., Huang, S.-Y., Fu, B.-Q., Liu, G.-Y., Chen, J.-X., Chen, M.-X., Yuan, Z.-G., Zhou, D.-H., Weng, Y.-B., Zhu, X.-Q. and Ye, D.-H., 2011. Seroprevalence of *Toxoplasma gondii* infection in pet dogs in Lanzhou, Northwest China. *Parasit. Vectors*, **4**: 64. https://doi.org/10.1186/1756-3305-4-64

Xiao, Y., Yin, J., Jiang, N., Xiang, M., Hao, L., Lu, H.,

Sang, H., Liu, X., Xu, H., Ankarklev, J., Lindh, J. and Chen, Q., 2010. Seroepidemiology of human *Toxoplasma gondii* infection in China. *BMC Infect. Dis*., **10**. https://doi.org/10.1186/1471-2334-10-4

Zhang, D., 2019. *Functional study of heparin binding proteins rop9, mic3 and SAG2 of* Toxoplasma gondii *and immunogenicity analysis of recombinant adenovirus*. Jilin University.

Zhao, X., Zhang, T., Sang, X., Yang, N., Feng, Y., Wang, Y., Chen, Q. and Jiang, N., 2017. Research and application progress of Toxoplasma gondii surface membrane antigen SAG1. *Chinese J. Pathog. Boil*., **12**: 697-699.

Zhou, J., Li, C., Luo, Y. and Wang, L., 2019. Antigenic epitope analysis and efficacy evaluation of GRA41 DNA vaccine against *T. gondii* infection. *Acta Parasitol*., **64**: 471-478. https://doi.org/10.2478/s11686-019-00091-3