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Establishment and application of a sandwich ELISA method for measuring *Toxoplasma gondii* circulating fructose-1,6-bisphosphate aldolase (ALD) protein in cats

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ABSTRACT

Toxoplasmosis is an important public health concern. Cats play a crucial role in increasing the risk of toxoplasmosis transmission to humans. Early diagnosis in cats is essential for the prevention and control of toxoplasmosis. In this study, we found that *T. gondii* aldolase (ALD) could be an effective diagnostic antigen, and then the recombinant ALD protein was expressed using the pET SUMO protein expression system, the mouse monoclonal antibody (MoAb) and rabbit polyclonal antibody (PoAb) of ALD were successfully produced, respectively. Furthermore, a reliable sandwich enzyme-linked immunosorbent assay (sELISA) was developed to detect circulating ALD in the sera of experimentally and naturally infected cats. rALD sELISA could detect *T. gondii* infection from 7DPI (post-infection day) to 14DPI with 100 % sensitivity and specificity, but could not detect *T. gondii* infection after 21DPI, indicating that it is a good early diagnosis tool. The detection limit was 7.8 ng/ml, the coefficients of variation (CV) of repeated tests within batches and between batches were confirmed to be less than 10 %. The results of 70 cat clinical serum samples detected by rALD sELISA were in almost perfect agreement beyond chance with those of a commercial ELISA kit (Cohen's kappa coefficient = 0.883). This sandwich ELISA method has high accuracy and can be used for early diagnosis of toxoplasmosis in cats.

1. Introduction

Toxoplasma gondii, an obligate intracellular parasitic protozoan, infects almost all warm-blooded animals, including livestock, birds and humans (Black and Boothroyd, 2000; Montoya and Liesenfeld, 2004). It is estimated that more than one-third of the global population is seropositive for *T. gondii*. While the majority of infected individuals remain asymptomatic; immunocompromised patients, pregnant women, elderly individuals, and children infected in utero are at serious risk of developing severe disease if exposed to the parasite (Jones et al., 2014). Cats (both domestic and wild felids) are the definitive hosts that can excrete the environmentally resistant oocysts (Di Genova et al., 2019). The global seroprevalence of *T. gondii* in domestic cats was estimated to be 35 % (95 % CI: 32–38 %) (Montazeri et al., 2020). Therefore, infection rates in cats are considered reliable indicators of *T. gondii* presence in the environment. Given the important public health implications, there is an urgent need to develop improved methods for the integrated control of toxoplasmosis in cats, including the use of specific and sensitive diagnostic tools.

Diagnosing toxoplasmosis based solely on clinical manifestations is challenging, as the symptoms and clinical signs are often nonspecific (Tenter et al., 2000). Common diagnostic methods for toxoplasmosis include pathogen detection techniques, such as direct microscopy for oocysts and trophozoites, animal infection tests, and molecular biology assays like polymerase chain reaction (PCR), gene sequencing, and isothermal amplification technology. However, both pathogen detection and molecular biology tests have limitations. For pathogen inspections, it's a challenge to isolate the parasites from circulation (Robert-Gangneux and Dardé, 2012), and microscopic examination is not easy to differentiate between *T. gondii*, *Hammondia hammondi*, or other parasite oocysts in cat feces (Dubey et al., 2020). Molecular biology tests, on the other hand, require specialized equipment and trained technicians, which limit their accessibility. Serological tests do not differentiate between species, but are often preferred for

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toxoplasmosis diagnosis, including direct or modified agglutination test (DAT/MAT), indirect hemagglutination test (IHA), and enzyme-linked immunosorbent assays (ELISA). Among them, ELISA is suitable for clinical use due to its ability to process large sample quickly, with high specificity and sensitivity. Multiple studies indicated that ELISA is a good diagnostic tool for toxoplasmosis in cats. For instance, GRA7 (dense granule antigen7) ELISA showed 89.7 % sensitivity and 92.5 % specificity for *T. gondii* infection in cats (Cai et al., 2015), and the SAG1 (surface antigen1) ELISA showed 100 % sensitivity and 96 % specificity (Hosseininejad, 2012). However, the specificity and sensitivity of ELISA depend on the antigens used, and further evaluation is needed to optimize these tests. Early diagnosis is crucial for instituting accurate therapy for toxoplasmosis and preventing mortality and morbidity (Jayshree and Mahadevan, 2022). For example, fetal disease is more severe when infection occurs early in pregnancy compared with later stages, early diagnosis plays an important role in warning pregnant women (Bollani et al., 2022). Additionally, infected cats typically shed oocysts for only 3–10 days after ingesting tissue cysts during acute infection (<14DPI). After the acute infection period, even cats with a positive serological diagnosis no longer shed oocysts (Hartmann et al., 2013). Since oocysts are extremely contaminating to the environment, early diagnostic methods for detecting acute infection in cats are important to control the transmission of toxoplasmosis. Sandwich ELISA for antibody detection serves as an effective tool for the early diagnosis of toxoplasmosis.

In our previous experiment (unpublished data), we identified some proteins that could serve as potential diagnostic markers for toxoplasmosis. In this study, we focused on one of them, fructose-1,6-bisphosphate aldolase (ALD), which belongs to the aldolase family. The importance of *T. gondii* ALD in glycolysis is well established (Tonkin et al., 2015), but is also essential for efficient host cell invasion (Starnes et al., 2009). These findings indicate that ALD may be a promising candidate for the development of diagnostic assays aimed at early identification of *T. gondii* invasion. We obtained highly reactive mouse monoclonal (MoAb 1C2) and rabbit polyclonal antibodies (PoAb) against recombinant ALD (rALD). The aim of the present study was to implement a sandwich ELISA (sELISA) for detecting circulating ALD antigen as a diagnostic tool for toxoplasmosis. We evaluated the performance of the method in both experimentally and naturally infected cats, demonstrating that the rALD sELISA is applicable for the early detection of toxoplasmosis in cats.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Animal Administration and Ethics Committee of

Yangzhou University, Yangzhou, Jiangsu Province China. All animals were handled strictly according to the Animal Ethics Procedures and Guidelines of the People's Republic of China, and the approval number of the animal experiments in this study was 202402120.

2.2. Animals and serum samples

Six to eight-week-old SPF (specific pathogen free)-grade female BALB/c mice (n = 5), eight to twelve -week-old female ICR mice (Institute of Cancer Research mice) (n = 3) and twelve -month-old female New Zealand rabbits (n = 3) were purchased from the Institute of Comparative Medicine, Yangzhou University. Cats (n = 10) were immunized subcutaneously with 3×10^6 inactive *T. gondii* RH tachyzoites, sera were collected at 7th, 14th and 21st day ($n_{7DPI} = 10$, $n_{14DPI} = 3$, $n_{21DPI} = 3$) after inoculation (DPI), and negative healthy cat serum samples (n = 10) were also collected. The field cat serum samples (n = 70) were kindly provided Yangzhou University Animal Hospital. All blood samples were collected from each animal aseptically into tubes without anticoagulant and serum was separated by centrifugation and preserved at −80°C for further use. To determine active infection, all cat serums were examined by the modified agglutination test (MAT).

2.3. Recombinant protein production

Constructing of plasmid pET23-SUMO-ALD (GenBank NO. AY150663.1) was performed via homologous recombination, following the published protocol (Pan et al., 2024). Briefly, the ald gene was amplified from cDNA of *T. gondii* RH strain using specific primers (forward 5'- ATGTCGGGATACGGT −3' and reverse 5'- CACGTAGCGTTTCTCG −3'), vector fragment was amplified from pET23-SUMO vector (pET23a-N-His SUMO Amp Vector) using specific primers (forward 5'-ACCGCGAACAGATTGGAGGTTCGGGATACGGTCTTCCCAT −3' and reverse 5'- GACCGGTACCACTAGTGGTCTTAGTACACGTAGCGTTTCT −3'). The plasmid pET23-SUMO-ALD, containing the corresponding homologous fragments, was constructed by multi-fragment cloning using the ClonExpress one-step cloning kit (Vazyme Biotech, Nanjing, China), and subsequently transfected into BL21 Rosetta (GenScript Biotech Corporation, Nanjing, China). The interested gene was confirmed by sequencing (GenScript Biotech Corporation, Nanjing, China). The recombinant ALD was induced by 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified using excel HisTrapTM affinity chromatography (GE Healthcare Life Sciences, USA). An additional purification step to remove SUMO fusion tags was modified using SUMO specific proteases Ulp1 according to (Yu et al., 2023).

2.4. The rALD immunoreactivity detection

Western blotting was used to analyze immunoreactivity of the recombinant protein. The proteins were separated by SDS-PAGE and then transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA), and blocked with PBS 5 % BSA (pH 7.4) overnight at 4°C. Anti-His antibodies (BBI Life Sciences Corporation, China), positive serum samples from cat and mouse diluted 1:200 in serum dilution buffer (PBS) were added respectively, and the membranes were incubated for 1 h at 37°C with shaking. After three washes with PBS-0.05 % Tween 20 (PBST), HRP-conjugated anti-mouse, anti-cat IgG (Sigma-Aldrich, USA), diluted 1:10,000 in blocking buffer respectively, were added and incubated for 1 h at 37°C, with shaking at 20 rpm. Finally, the bands were revealed using ECL solution (Tanon Life Science Corporation, Shanghai, China).

2.5. Production of monoclonal antibody (MoAb) against rALD

Obtaining MoAbs against rALD were modified from previous study (Anuracpreeda et al., 2016). Briefly, MoAbs against rALD were produced by fusion of mouse myeloma cells (SP2/0) with the spleen cells from BALB/c mice immunized rALD, using polyethylene glycol (Sigma-Aldrich, USA) as the fusion agent. The resulting hybridoma cells were screened for their ability to recognize rALD using an indirect ELISA. The highly reactive hybridoma cells were then cloned though limiting dilution methods. Antibody isotypes were screened by an iELISA kit (Biodragon, Suzhou, China). One hybridoma clone, designated 1C2, was selected for producing a high titer of antibody against rALD.

2.6. Assessing specificity of MoAb 1C2

Immunoblotting assay was used for assessing the reactivity of MoAb. *Toxoplasma* lysate antigen (TLA, 10 μg, our lab) and recombinant cathepsin L7 from *Fasciola hepatica* (10 μg, our lab) were separated by 12 % SDS-PAGE, and then transferred onto PVDF membrane. Then the PVDF membrane was incubated in diluted MoAb 1C2 (1:1000) at room temperature for 1 h. Finally, bound MoAb 1C2 on the PVDF membrane was detected by incubation with HRP-conjugated anti-mouse-IgG (Sigma-Aldrich, USA.). The bands were revealed using ECL solution

(Tanon Life Science Corporation, Shanghai, China).

2.7. Purification of MoAb 1C2 against rALD

The IgG fraction of MoAb was purified using 50 % saturated ammonium sulfate precipitation, followed by dialysis against excess PBS. The purified IgG was then applied to an affinity chromatography column containing Mab trap protein G Sepharose (GenScript Biotech Corporation, Nanjing, China) (Anuracpreeda et al., 2016).

2.8. Production of polyclonal antibody (PoAb) against rALD

PoAb against rALD was prepared by immunizing New Zealand White rabbits. Briefly, 50 μg rALD was mixed with adjuvant (Biodragon, Suzhou, China), and injected intramuscularly into the rabbits. Two booster injections were administered at 3-week intervals. Blood samples were collected 1-week after final boost and tested for the antibody titers.

2.9. Set up sELISA

The optimal conditions for the sELISA were determined through a series of experiments. The microplates (Corning Costar, US) were coated with different concentrations (0.625, 1.25, 2.5, 5 μg/ml) of purified MoAb 1C2 in carbonate buffer (0.1 ml of 0.05 M $NaHCO_3$, pH 9.6), and incubated overnight at 4°C or 2 h at 37°C. After washing with (×3) PBST (PBS with 0.05 % Tween-20), the plates were blocked with 3 % BSA for 2 h at 37 °C. rALD (0.5 μg in 100 μl PBS) or serum samples were incubated with each testing well for 1 h at 37 °C, followed by five washes with 0.05 % PBST (pH 7.4). Plates were then incubated with PoAb (1.825, 3.75, 7.5, 15 μg/ml, 100 μl/well) and HRP-conjugated-anti-rabbit IgG (Sigma-Aldrich, USA, 1:10,000, 100 μl/well) for 1 h at 37 °C. After washing steps (×3), 100 μl/well of TMB (Beyotime Biotechnology, Shanghai, China) was added and incubated at 37 °C for 10 min. The reaction was stopped by 50 μl/well 2 M H_2SO_4. The optical density was determined at a wavelength of 450 nm ($OD_{450\ nm}$) using a microplate reader (Sunrise-basic Tecan, Germany).

The optimal ELISA protocol was finally determined. The optimal coating concentration of MoAb 1C2 was 5 μg/ml incubated overnight at 4°C. After washing with (×3) PBST, the plates were blocked with 3 % BSA for 2 h at 37 °C. The serum samples were diluted 1:100 in PBS (pH 7.4) and then incubated with each testing well for 1 h at 37 °C. After washing with (×5) PBST, Plates were then incubated with PoAb (1.825 μg/ml, 100 μl/well) and HRP-conjugated-anti-rabbit IgG (Sigma-Aldrich, USA 1:10,000,100 μl/well) for 1 h at 37 °C. After washing steps (×3), 100 μl/well of TMB (Beyotime Biotechnology, Shanghai, China) was added and incubated at 37 °C for 10 min. The reaction was stopped by 50 μl/well 2 M H_2SO_4. To evaluate the detection limit of sELISA, the rALD was serially diluted (16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0156, 0.0078, 0.0039,0.001953 μg/ml) in PBS containing 1 % healthy cat serum and tested. The OD values were examined and correlated with the respective amounts of antigen. The endpoint of detection limit was defined as the lowest amount of antigen that still produced positive OD values. To estimate the inter-and intraassay precision, the coefficients of variation (CVs) of repeated tests were calculated within and between batches, were calculated based on three replicates of each sample using positive (n = 5) and negative (n = 2) cat serum samples.

2.10. Clinical sample analysis

A total of 70 cat serum samples, collected from Jiangsu China, were examined using the established rALD sELISA method. In parallel, a commercial ELISA kit (Haitai, China) was applied to detect these cat serum samples for the comparative test.

2.11. Statistical analysis

Statistical analysis was conducted using the Graphpad 6.01 software. The mean OD values of standard positive and negative serum were compared using one-way ANOVA test, and P values < 0.05 were considered to show statistical significance. The optimal cut-off value for ELISA method was established within a 95 % confidence interval (CI), by receiver operating characteristic (ROC) curve analysis (Swets, 1988), with the maximum value for Youden's J (Youden's J =Sensitivity + Specificity −1). The degree of agreement between the methods and the commercial ELISA kit was quantified using Cohen's kappa coefficient, also performed using the Graphpad 6.01 software (Landis and Koch, 1977).

3. Results

3.1. Expression, purification, and immunoreactivity of rALD

To optimize the expression conditions of rALD, different temperatures and induced time were evaluated in this study. The optimum expression condition was the BL21 (Rosetta) induced for 4 h at 37°C by 0.5 mM IPTG (Fig. 1A). SDS-PAGE analysis revealed the presence of an approximately 55 kDa protein, consistent with the expected size of SUMO-rALD. Most of rALD was expressed in a soluble protein form under different conditions (Fig. 1A lanes 3&6). Purified protein was shown as a single band at the expected molecular weight (~55 kDa) by SDS-PAGE (Fig. 1B). The result of western blotting indicated that the purified SUMO-rALD could be specifically recognized by anti-His tag antibody (Fig. 1C lane 3), and also be recognized by the positive serum from *T. gondii* infected cat and mouse. These results indicated that rALD had reactions with different positive sera, indicating good immunoreactivity and its potential as a diagnostic candidate for toxoplasmosis (Fig. 1C lane 1 & lane 2). The additional purification step was modified to remove SUMO fusion tags using SUMO specific proteases Ulp1. These results indicated that SUMO had been successfully removed by Ulp1, with the resulting protein exhibiting an approximate size of 39 kDa, consistent with the expected size of rALD (~39 kDa) (Fig. 1D lane 2).

3.2. MoAbs against rALD

Three stable hybridoma clones of MoAbs against rALD, designated 1C2, 2H12, 4D1, were produced. These clones were selected and expanded in culture flasks to obtain a large volume of MoAbs, which were then collected for further experiments. The immunoglobulin isotypes were determined, with 1C2 and 2H12 classified as IgG1, and 4D1 classified as IgG2. Clone 1C2 had the highest titer, was used in this study. The immunoblotting assay (Fig. 2A) were used for studying the specificity of MoAb 1C2. MoAb 1C2 exhibited a strong reaction with *T. gondii* RH TLA antigen at molecular weight (MW) of 39 kDa, while showing no cross-reaction with *Fasciola* antigens from same *Escherichia coli* expression system. Purity of the MoAb 1C2 was assayed by 12 % SDS-PAGE, which revealed that the purified IgG consisted of heavy and light chains at molecular weights of approximately 53 kDa and 25 kDa, respectively. IgG appeared free from any other proteins (Fig. 2B).

3.3. Optimizing sELISA and ROC analysis of rALD sELISA

The results indicated that the optimal coating concentration of MoAb 1C2 was 5 μg/ml incubated overnight at 4°C, with the serum dilution of 1:100 and PoAb 1.825 μg/ml. Under the condition, cat sera from acute infection ($n_{7DPI} = 10$, $n_{14DPI} = 3$) were used for further study, the result indicated that the cut-off value was 0.3745 based on ROC curve analysis according to the maximum value for Youden's J (Fig. 3A). The absorbance values for positive cat sera ranged from 0.5910 to 0.7000 (0.6198 ± 0.2968, $n_{7DPI,\ 14DPI} = 13$), while the values for the negative control group ranged from 0.1340 to 0.1580 (0.1340 ± 0.1580, n = 10). Both

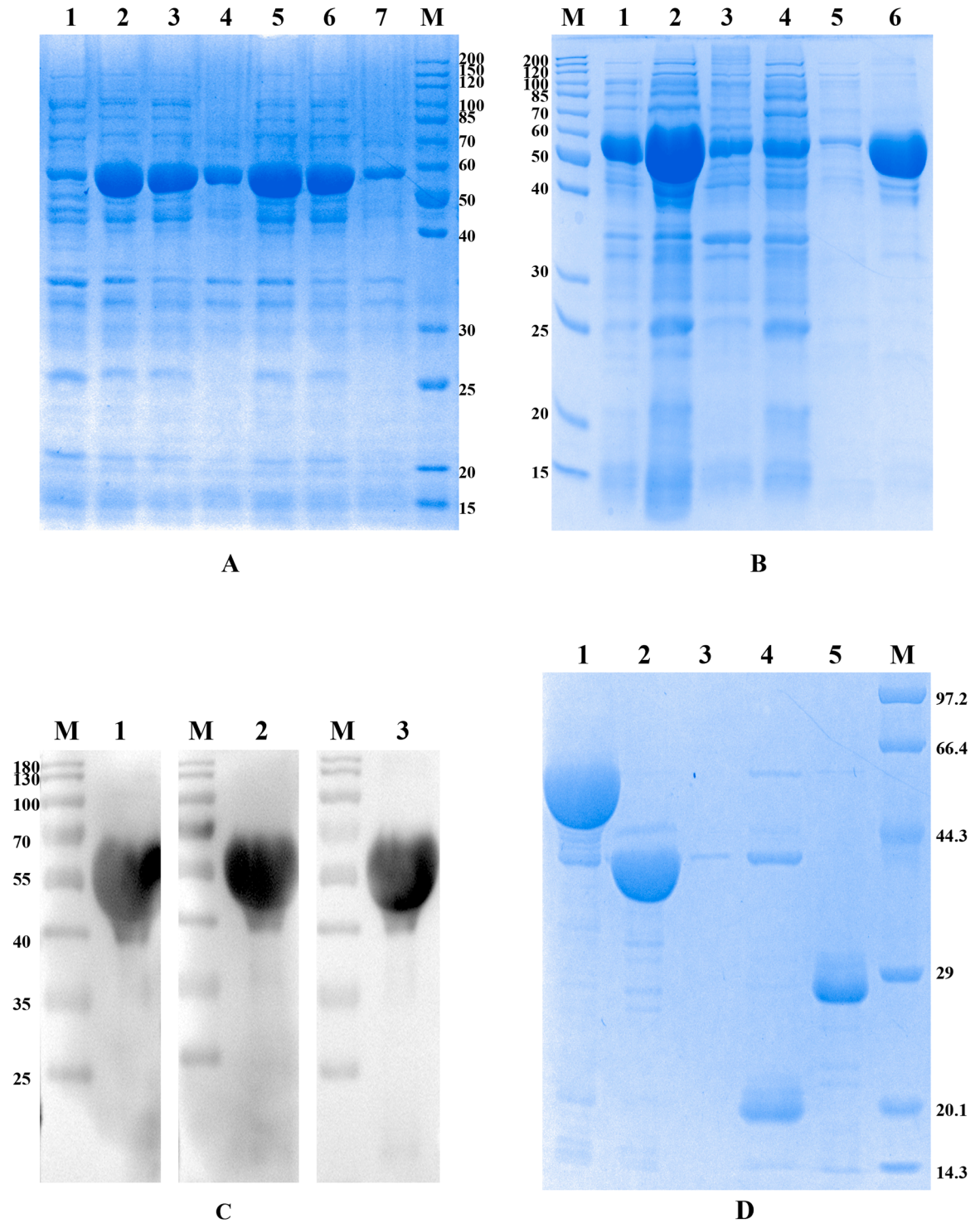

Fig. 1. Optimization, Expression, Purification, and Immunoreactivity Evaluation of rALD. (A) The expression of rALD in *E. coil* BL21 Rosetta at 37°C and 25°C analyzed on 12 % SDS-PAGE. Lane 1: non- induced; lanes 2&5: indued by IPTG; lane 3&6: supernatant; lanes 4&7: the pellet. Lanes 2–4 were induced at 37°C and lanes 5–7 were induced at 25°C. (B) Purified protein rALD resolved by 12 % SDS-PAGE. Lane M: Molecular mass marker (Thermo Fisher Scientific); lane 1: induced SUMO-rALD Rosetta; lane 2: induced supernatant; lane 3: induced precipitation; lane 4: Flow through fluid; lane 5: washing steps; lane 6: Purified SUMO-rALD (~55KDa). (C) SUMO-rALD were analyzed by 12 % SDS-PAGE, transferred onto PVDF membrane and then probed with positive cat serum (lane 1), positive mouse serum (lane 2), and anti-HIS-tag antibody (lane 3). Prestained Protein Ladder (lane M Thermo Fisher Scientific). (D) Digestion of SUMO-rALD by Ulp1 was shown by 12 % SDS-PAGE. LaneM: Premixed Protein Marker (Low Molecular mass, TaKaRa), lane 1: Unpurified and undigested SUMO-rALD (~55KDa), lane 2: rALD digested by Ulp1(~39KDa), lane 3: Washing buffer, lane 4: SUMO-HIS Tag in Elution Buffer (~20KDa), lane 5: Ulp1 (~26KDa).

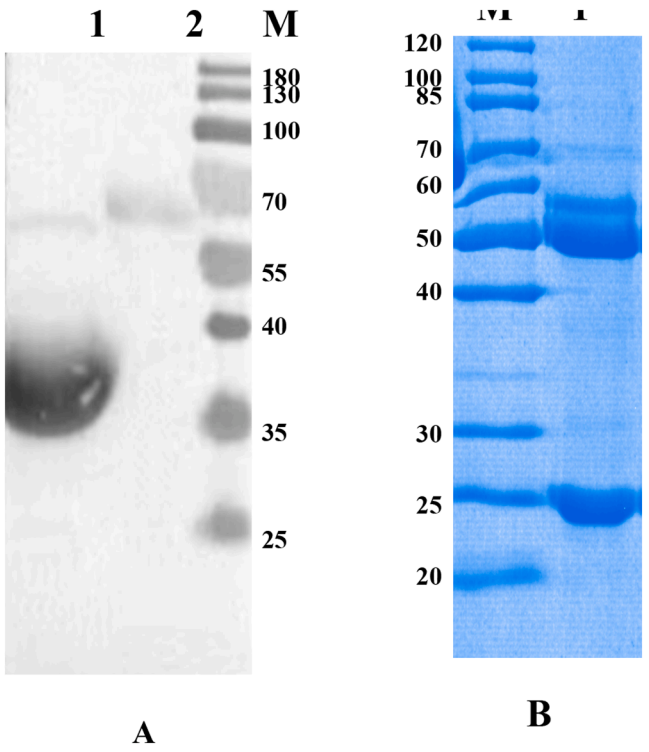

Fig. 2. Immunoreactivity Evaluation and Purification of MoAb 1C2. (A) *T. gondii* TLA (10 μg, lane 1) and recombinant cathepsin L7 of *Fasciola hepatica* (10 μg, lane 2) were separated by 12 % SDS-PAG, transferred onto PVDF membrane and then probed with MoAb 1C2. (B) Purified MoAb 1C2 was represented by H- and L-chain at 53 and 25 kDa, respectively. IgG appeared free from any other proteins.

sensitivity and specificity were found to be 100 %, with an area under the curve (AUC) value of 1.0. To evaluate rALD sELISA's ability to distinguish between acute and chronic infection, sera from chronic infection periods (21DPI) were also tested, A significant difference was observed between the OD450 nm values of negative control group and all infected groups (7DPI, 14DPI and 21DPI, $P < 0.0001$), but OD450 nm values from cat serum at 21DPI was below the cut-off value 0.3745 (Fig. 3B).

3.4. The detection limit and intra-and inter-assay precision of rALD sELISA

The ELISA cut-off value was 0.3745. The detection limit of this assay was assessed using various concentrations of rALD, as illustrated in Fig. 4A. This method could detect rALD at the lowest concentrations of 7.8 ng/ml. Regarding the intra- and inter-assay precision, the coefficient of variation (CVs) for repeated tests within batches and between batches were confirmed to be less than 10 % (Fig. 4B), indicating the good stability of rALD sELISA.

3.5. Clinical application of rALD sELISA

70 clinical cat samples from Yangzhou University Animal Hospital were examined by rALD sELISA. The overall seroprevalence was 14.3 % (10/70). In parallel, a commercial ELISA kit also indicated a positive result of 14.3 % (10/70). One sample identified as positive by the commercial kit tested negative for rALD sELISA, while one sample identified as negative by commercial kit tested positive for rALD sELISA. There was an almost perfect agreement between the rALD sELISA and the commercial ELISA kit, with an agreement proportion of 97.1 % (agreement beyond chance, Kappa value: 0.883; 95 %CI: 0.725–1.000) (Table 1).

4. Discussion

T. gondii is an obligate intracellular apicomplexan parasite, which need invade host cells to survive. Previous studies reported that the fructose-1,6-bisphosphate aldolase (ALD) played a crucial role in transmitting the mechanochemical force generated by the motor to the extracellular adhesion during the invasion stage of *T. gondii* (Boucher and Bosch, 2014). In addition, ALD can bind polymerized actin in vitro (Tonkin et al., 2015), and has been identified primarily important for energy metabolism in *T. gondii*, ALD-depleted parasites had impaired growth in glucose (Shen and Sibley, 2014). *T. gondii* apical membrane antigen 1 (AMA1), a microneme protein, binds to ALD and may participate in parasite invasion (Shen and Sibley, 2014). Previous research reported that indirect ELISA (iELISA) based on AMA1 had the higher overall check accuracy (sensitivity: 91.7 %, specificity: 93.6 %) comparing to GRA7 iELISA (sensitivity: 91.7 %, specificity: 85.5 %) in cats (Gao et al., 2023). Therefore, ALD is an important functional protein of *T. gondii,* similar to AMA1, and may serve as a better diagnostic target compared with AMA1 or GRA7.

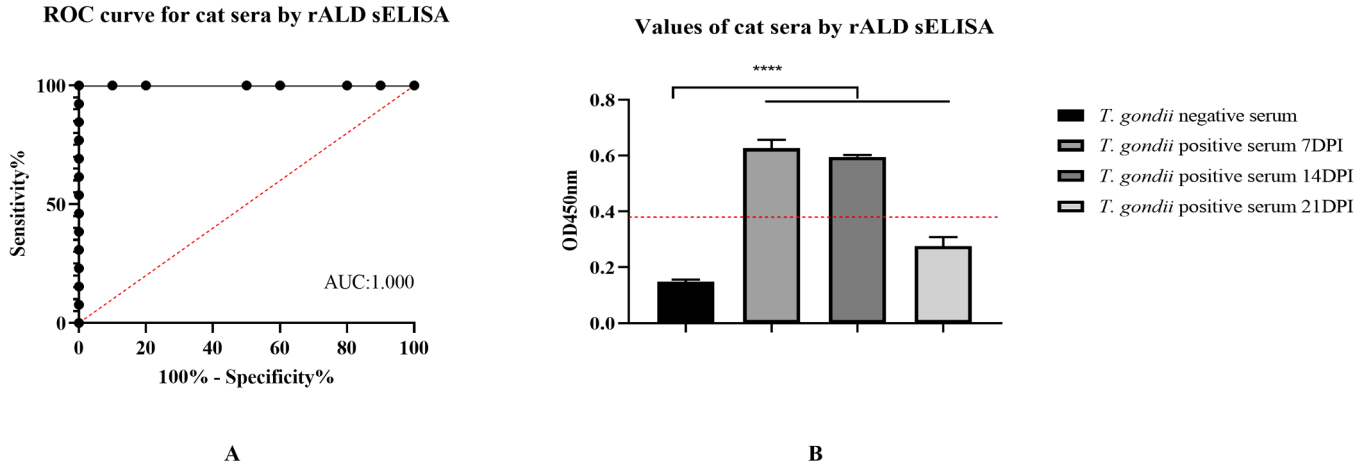

Fig. 3. Receiver-Operator Characteristic (ROC) Curve and Box Plot Showing $OD_{450\ nm}$ Values of Cat Sera Detected by rALD sELISA. The ROC was used to evaluate the ability of rALD sELISA to distinguish between *T. gondii*. infected cats (7DPI 14DPI) versus healthy cats (area under curve AUC=1.00; $P < 0.0001$) (A). The cut-off value was 0.3745, and 100 % diagnostic sensitivity and specificity at Youden's J max value. (B) The box plot included absorbance values of ELISA results for positive ($n_{7DPI,14DPI\ and\ 21DPI} = 16$) and negative groups ($n = 10$) showed significant differences by one-way ANOVA test ($P < 0.0001$).

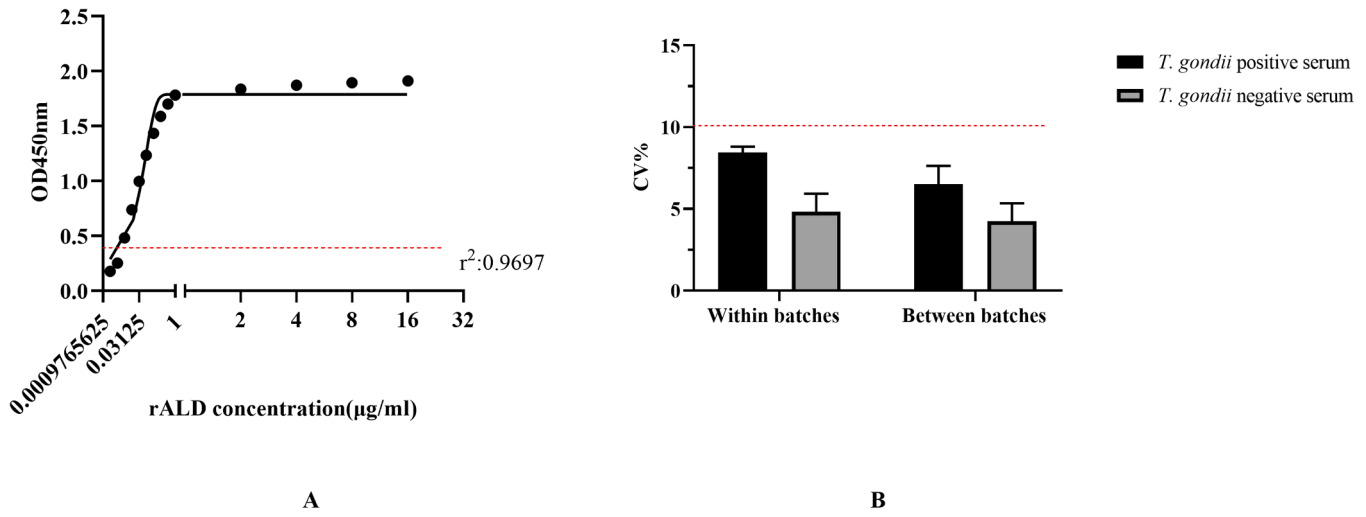

Fig. 4. Detection Limit and Coefficient of Variation (CV) of rALD sELISA. (A) The lowest detection limit of rALD sELISA was determined by serial dilutions of rALD using Sigmoidal, 4PL (Graphpad 6.01, r^2:0.9697). (B) CV of repeated tests, both within and between batches, was calculated based on three replicates of each sample (positive n = 5, negative n = 2).

Table1
Detection of *Toxoplasma gondii* infection in cats by rALD sELISA in comparison with commercial ELISA kit [a].

Detection method		rALD sELISA		
		Positive	Negative	Total
Commercial ELISA kit	Positive	9	1	10
	Negative	1	59	60
	Total	10	60	70

[a] The degree of agreement was quantified using Kappa statistics using the Graphpad 6.01 software. Kappa < 0: No agreement, Kappa between 0.00 and 0.20: Slight agreement, Kappa between 0.21 and 0.40: Fair agreement, Kappa between 0.41 and 0.60: Moderate agreement, Kappa between 0.61 and 0.80: Substantial agreement, Kappa between 0.81 and 1.00: Almost perfect agreement.

In our previous experiment (unpublished data), the cats were infected *T. gondii*, and sera were collected from different periods (7DPI, 14DPI, and 21DPI). The interaction proteins were pulled down using these sera and identified by LC-MS/MS. ALD was one of these proteins that interacted with sera. The fusion tags can enhance solubility and expression efficiency of recombinant protein. To obtain soluble rALD, we utilized the SUMO expression system, successfully improved the quality of ALD in this study (Fig1). Other studies have similarly reported the successful expression of genes using comparable expression systems (Prejit et al., 2019; Tan et al., 2020). For example, the pETite™ vector with an N-terminal 6xHis SUMO fusion tag, was used to enhance the solubility and expression level of GRA7 (Suwan et al., 2022). In addition, ULP1 was used to specifically cleave the SUMO fusion tags to obtain high purity of rALD. This is particularly important for the production of more specific anti-ALD -monoclonal and polyclonal antibodies and the detection of complex cat serum antibodies.

Cats can transmit *T. gondii* to humans and livestock by excreting resistant oocysts in feces. Infected cats typically shed oocysts for only 3–10 days after ingesting tissue cysts during acute infection (Dubey et al., 1970; Hartmann et al., 2013). Thus, the definitive diagnosis prior to oocyst release is essential. *T. gondii* IgG avidity assay has been used as a standard diagnostic technique, but has limitations to detect the acute infection phase (Dubey et al., 2020). Sandwich ELISA, which detects antigens rather than antibodies, is considered to be a more accurate method. At present, sELISAs for *T. gondii* primarily were total circulating antigen (CAg) detection kits. Studies have shown that the earliest positive detection using CAg sELISA occurred at 3 DPI in rabbits, with a lower detection limit of 31.2 ng/ml (Chen et al., 2008). In Shanghai, the positive value of *T. gondii* in cats was reported at 5.5 %, indicating that CAg detection is necessary in surveys of *T. gondii* infection in cats (Wang et al., 2012). Similarly, rALD sELISA was able to detect circulating ALD antigen in cats as early as 7DPI in this study, but could not detect 21DPI serum (cut-off:0.3745). rALD sELISA effectively distinguish acute infection from chronic infection. Furthermore, the rALD sELISA demonstrated a detection limit of 7.8 ng/ml, which was better than the performance of CAg sELISA. Collectively, these results suggest that rALD could be a sensitive and specific marker for acute infection, which will help to control the transmission of toxoplasmosis.

In recent years, the rate of *T. gondii* infection in domestic cats has been reported, with the positive rate of 19.9 % in mainland China and 35 % globally (Gao et al., 2023; Montazeri et al., 2020; Zhou et al., 2022). The higher seroprevalence rate in cats is closely related to their ecological habits (Foroutan et al., 2019). Yangzhou City (32.15″-33.25″ N, 119.01″-119.54″ E), is located in central Jiangsu Province, at the junction of the Yangtze River and the Beijing-Hangzhou Grand Canal. Consequently, water contaminated by oocysts excreted from *T. gondii* infected cats may facilitate rapid transmission on a large scale. In this study, all clinical samples were obtained from domestic cats, which showed a positive rate of 14.3 % using both the rALD sELISA and a commercial ELISA kit (Table 1). There was an almost perfect agreement between the rALD sELISA and the commercial ELISA kit. This finding is consistent with the previous infection rate of 22 % in cats in Jiangsu Province (Zhou et al., 2022). These results suggest that pet cats in Jiangsu are at risk of *T. gondii* infection. rALD sELISA, being a convenient and quick detection method, may be an effective tool for diagnosing toxoplasmosis in cats, thereby protecting owners especially the pregnant from infection.

5. Conclusion

In this study, a novel diagnostic antigen, ALD, was expressed and interacted with sera from *T. gondii*-positive cats. The results demonstrated that the rALD MoAb-PoAb-based sandwich ELISA (sELISA) was a reliable method for detecting acute infection in cats, exhibiting 100 % sensitivity and specificity, repeatability, and a low detection limit. The rALD sELISA could detect the infected cat as early as 7DPI. This method may serve as a valuable tool for diagnosis of *T. gondii* infection in cats.

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CRediT authorship contribution statement

Yun-Ping Wu: Writing – original draft, Project administration, Methodology, Formal analysis, Conceptualization. **Ming Pan:** Writing – original draft, Investigation, Conceptualization. **Zhao-Feng Hou:** Writing – review & editing, Funding acquisition. **si yang Huang:** Writing – review & editing, Resources, Funding acquisition. **Jing-Zhi Gong:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Yi-Min Fan:** Writing – original draft, Visualization, Validation, Project administration, Methodology, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Si-Yang Huang reports financial support was provided by Outstanding Youth Foundation of Jiangsu Province of China. Si-Yang Huang reports financial support was provided by Priority Academic Program Development of Jiangsu Higher Education Institutions. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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