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RESEARCH

Identification of specific antigens between *Toxoplasma gondii* and *Neospora caninum* and application of potential diagnostic antigen TgGRA54

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Abstract

Toxoplasma gondii is a zoonotic parasite that is very common in livestock. Meat products from livestock infected with *T. gondii* are one of the important transmission routes of toxoplasmosis. Rapid and reliable diagnosis is a prerequisite for the prevention and control of toxoplasmosis. *Neospora caninum* and *T. gondii* are similar in morphology and life history, and there are a large number of cross antigens between them, making clinical diagnosis of toxoplasmosis more difficult. In this study, immunoprecipitation-mass spectrometry (IP-MS) was used to screen for *T. gondii*–specific antigens, and the specific antigen was cloned and expressed in *Escherichia coli*. The specific antigen was then used to establish an indirect ELISA diagnostic method. A total of 241 specific antigens of *T. gondii* and 662 cross antigens between *T. gondii* and *N. caninum* were screened by IP-MS. Through bioinformatics analysis and homology comparison, seven proteins were selected for gene cloning and prokaryotic expression, and the most suitable antigen, TgGRA54, was selected to establish an indirect ELISA for *T. gondii*. Compared with the indirect immunofluorescent antibody test (IFAT), the positive coincidence rate of the ELISA based on rTgGRA54 was 100% (72/72) and the negative coincidence rate was 80.95% (17/21). The indirect ELISA method based on TgGRA54 recombinant protein was established to detect *T. gondii* antibodies in bovine sera, and the recombinant protein reacted well with *T. gondii* positive sera from sheep, mouse, and swine, indicating that the recombinant protein is a good diagnostic antigen for *T. gondii*.

Keywords *Toxoplasma gondii* · Specific antigen · Diagnostic antigen · TgGRA54 · Indirect ELISA

Introduction

Toxoplasma gondii is an apicomplexan intracellular protozoan parasite that infects all warm-blooded vertebrates, including humans and domestic animals (Robert-Gangneux and Dardé 2012). Its complete life cycle requires two hosts, with intermediate hosts including mammals, some birds, and final hosts being cats and other felines. *T. gondii* reproduces sexually in the intestine of cats, producing millions of cysts known as oocysts, which are excreted in the feces (Dubey 2004, 2009). The ingestion of oocysts with contaminated food or water can infect humans and other warm-blooded animals. In addition, humans can be infected by consuming meat from animals containing *T. gondii* tissue cysts (Dubey et al. 1998). Based on epidemiological studies, consumption of uncooked or undercooked beef can be a risk factor for the transmission of *T. gondii* to humans (Baril et al. 1999; Cook et al. 2000). As the meat industry is of great economic importance

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worldwide, the infection of meat-producing animals, such as cattle, with *T. gondii* can pose a threat to human health and will also have a negative impact on human socio-economics (van der Puije et al. 2000).

However, to date, there is no effective drug or vaccine available to prevent and cure toxoplasmosis, which makes the diagnosis of *T. gondii* particularly important. The screening of diagnostic antigens of *T. gondii* is of great significance for the diagnosis of *T. gondii*.

Although *N. caninum* and *T. gondii* differ considerably in terms of host range and pathogenicity, they both are specific intracellular parasites belonging to the subclass coccidia having similarities in morphological structure, genome, transcriptome level, and life history with many identical protein compositions (Nishikawa et al. 2002; Reid et al. 2012). This has led to many misdiagnoses in the clinical diagnosis of toxoplasmosis. The high similarity between the two pathogens has always been an important obstacle to the accurate clinical diagnosis of *T. gondii*. The search for valuable specific diagnostic antigens of *T. gondii* is of great significance for the diagnosis of *T. gondii*.

In the past 20 years, a large number of recombinant proteins, including the dense granular proteins GRA1, GRA2, GRA4, GRA5, GRA6, GRA7, and GRA8 (Lau et al. 2012; Wang et al. 2014a, 2014b); the rhoptry proteins ROP1 and ROP2 (Chang et al. 2011); the stromal antigen MAG1; the microneme proteins MIC2, MIC4, and MIC5; and the surface antigens SAG1 and SAG2 (Thiruvengadam et al. 2011), have been studied and applied to detect toxoplasmosis in humans or animals. These antigens have potential diagnostic value in evaluating ELISA detection of specific IgG and IgM antibodies. However, there are many cross-reactive antigens between *T. gondii* and *N. caninum*, leading to many misdiagnoses, such as MIC3 (Yang et al. 2015). Besides, the detection efficiency of different diagnostic antigens at different stages varies, and it is difficult to evaluate *T. gondii* infection with a single diagnostic antigen. Previous studies have shown that the combination of different diagnostic antigens can effectively improve the detection effect, so the exploration of new diagnostic antigens and combining them with other diagnostic antigens may be considered in clinical diagnosis to obtain more accurate detection information (Holec-Gąsior et al. 2014). Moreover, the identification of specific antigens and cross antigens of *T. gondii* and *N. caninum* is of great significance for exploring the common mechanisms of parasite-host interactions and improving serological diagnosis.

In this study, IP-MS was used to screen for *T. gondii*–specific diagnostic antigens, and a variety of prokaryotic expressed proteins of *T. gondii* were prepared to establish a method for further detection of *T. gondii* and evaluate their diagnostic potential in ELISA.

Materials and methods

Parasites and cell cultures

T. gondii RH strain tachyzoites were propagated in African green monkey kidney (Vero) cells, which were cultured in Dulbecco's modified Eagle's medium (DMEM) (M&C, China) containing 25 mM glucose and 4 mM glutamine and supplemented with 2% fetal bovine serum (FBS, Gibco, USA). Cells were incubated at 37 °C with 5% CO_2 in a humidified incubator.

Acquisition of *T. gondii* cysts and preparation of positive serum

Five 4-week-old female BALB/c mice (Peking University Health Science Center, China) were selected, and each mouse was injected intraperitoneally with 1×10^5 *T. gondii* PRU tachyzoites to induce chronic infection. One month later, brain tissue was ground and collected, and cysts were counted microscopically. Ten 4-week-old female BALB/c mice were then infected with 4 cysts per mouse by intragastric administration, and blood was collected from these mice after 1 month of infection. Five 4-week-old BALB/c mice were used to prepare *N. caninum*–positive serum by intraperitoneal injection of 1×10^5 *N. caninum* tachyzoites per mouse, and blood was collected from these mice after 1 month of infection. The animals were housed under conditions free of specific pathogens with ad libitum access to feed and water.

Preparation of soluble *T. gondii* lysate antigen

Tachyzoites were harvested from Vero cell cultures. Twenty-seven-gauge needles were used to disrupt the cells, and the lysates were filtered through a 5-μm syringe filter. Purified tachyzoites were washed three times in DMEM by centrifugation at 900 × g for 10 min. Freshly purified tachyzoites were incubated (5×10^7 parasites/mL) in a serum-free medium (DMEM) at 37 °C for 3 h and then cooled on ice for 10 min. The parasites were lysed using a RIPA buffer (Beyotime, China) supplemented with a cocktail of protease inhibitors (Sigma, USA). The supernatant was collected, and the protein concentration was determined by BCA assay using BSA as the standard.

Immunoprecipitation-mass spectrometry

Ten microliters of protein A/G beads was taken and washed three times with PBS, and incubated with 5 μL *T. gondii*–positive serum or and *N. caninum*–positive serum reapectively.

Then the binding products was incubated with *T. gondii* lysate antigen. The mixture was further incubated at 4 °C for 2 h. The beads were washed 3 times with buffer (20 mM Tris-HCl, pH 6.8, 150 mM NaCl, 0.2% TritonX-100), resuspended in 2×SDS loading buffer and cooked at 95 °C for 5–10 min and then analyzed by SDS-PAGE. Peptides were dissolved in 0.1% formic acid and subjected to LC-MS/MS analysis (QLbio, China).

Bioinformatics analysis

The nucleic acid sequence and amino acid sequence of the selected proteins were searched according to the gene number in the database (ToxoDB, http://www.toxoDB.org, Ver.8.2), and bioinformatics information such as signal peptide and transmembrane domain was recorded. The amino acid sequences of the selected proteins were compared in the NCBI database (https://www.ncbi.nlm.nih.gov/).

Gene cloning, recombinant protein expression, and purification

According to the ToxoDB database (http://www.toxodb.org/toxo/) of *T. gondii*, the bioinformatics, homology, and transcriptional level of *T. gondii* proteins were analyzed and the nucleotide sequences of TGME49_247520, TGME49_328700, TGME49_290700, TGME49_204340, TGME49_253690, TGME49_237015, and TGME49_273130 proteins of *T. gondii* were selected for gene cloning and prokaryotic expression.

Total RNA was extracted from purified *T. gondii* tachyzoites, reverse-transcribed (TransGen Biotech Co., Ltd., China), and used as a template for gene amplification.

DNA extraction kit (Aidlab Biotechnologies Co., Ltd., Beijing, China) was used to extract genomic DNA from the RH strain. All primers used in this study are listed in Table S1. The pET-28a vector (Novagen, Madison, Germany) was inserted with the complete coding sequence and transformed into *Escherichia coli* BL21 (Transetta, TansGen Biotech Co., Ltd., Beijing, China) for recombinant protein expression. The proteins were purified by affinity chromatography using Ni-IDA agarose in accordance with the manufacturer's standard protocol. Expressed and purified recombinant proteins were assessed by SDS-PAGE analysis. The gel consisted of running gel (12%, w/v, acrylamide) and stacking gel. The electrophoresis buffer was 25 mM Tris, 192 mM glycine, 0.1% SDS, and pH 8.3. After electrophoresis, the gels were stained using Coomassie brilliant blue (Thermo Fisher Scientific Inc., Waltham, MA, USA) for protein detection. They were treated overnight in protein destaining solution (10% acetic acid, 30% methyl alcohol, 60% distilled water, v:v:v). The relative molecular mass of each of the recognized bands was determined by comparison with standard markers.

Western blot analysis

The rTgGRA54 was subjected to electrophoresis and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). After being blocked with 5% (w/v) powdered milk (BD Difco, USA), the blots were probed with mouse anti-his antibody (Sigma-Aldrich) at a dilution of 1:500 for 1 h. After five washes with PBST (1% Tween-20), the membranes were incubated with 1:5000 diluted HRP-conjugated goat anti-mouse IgG (Sigma, USA) for 1 h, washed, and visualized using Plus ECL (CoWin Biotech Co., Ltd, China).

The reactivity of rTgGRA54 with *T. gondii* antibody–positive sera from other animals was analyzed by western blot. The primary antibody was used with *T. gondii* antibody–positive/negative sera from mouse, sheep or swine. The method is the same as above.

Indirect ELISA

Optimal dilutions of the antigen and bovine sera were determined by checkerboard titration. *T. gondii*–positive and *T. gondii*–negative sera samples were employed for each assay. We diluted the his-fused rTgGRA54 in a coating buffer (0.02M Tris-HCl buffer, pH 7.2) to a final concentration of 0.25 μg/well, added it to 96-well flat-bottom plates (Guangzhou Jet Bio-filtration Co., Ltd., China), and incubated it at 37 °C for 1 h and then at 4 °C overnight. After washing four times with a washing buffer (PBS containing 0.1% Tween 20), the plates were blocked with a blocking buffer (PBS containing 5% horse serum) at 37 °C for 1 h. The plates were washed four times, the bovine sera were diluted in a diluent solution (PBS containing 2% horse serum, 1:200), and 100 μL was added to each of the duplicate wells of the ELISA plate and incubated for 1 h at 37 °C. The plates were rinsed as described previously and incubated with HRP-conjugated goat anti-bovine IgG antibody (Southern Biotechnology Associates, Inc., USA) diluted in a diluent solution (1:25,000) at 100 μL/well for 0.5 h at 37 °C. Finally, the plate was rinsed and bound antibodies were detected by incubating with 100 μL/well of tetramethylbenzidine (TMB) substrate (M&C Gene Technology Co., Ltd., China) with color rendering at room temperature for 5 min. The reaction was terminated with a stop solution (2 M sulfuric acid, 50 μL/well), and the absorbance was measured at 450 nm in an ELISA plate reader (Bio-Rad). Each experiment was repeated three times. The cutoff point was determined as the mean OD450 of *T. gondii*–negative sera kept in our laboratory ($n = 24$) plus three standard deviations. Samples with an OD value ≥ cutoff point were considered as positive for *T. gondii*, and those with an OD value < cutoff point were considered as negative for *T. gondii*.

Data analysis

Statistical analysis of the data was performed using SPSS 22. Kappa coefficient was used to evaluate the level of agreement between ELISA methods (based on rTgGRA54 or rTgSAG1) and the gold standard (IFAT). The specificity and sensitivity for the detection of *T. gondii* serum antibodies by ELISA were determined using the following formulas: sensitivity = (number of ELISA tested-positive sera/number of IFAT tested-positive sera) × 100%; specificity = (number of ELISA tested-negative sera/number of IFAT tested-negative sera) × 100%).

Ethical statement

Animal experiments in this study were performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China and approved by the Institutional Animal Care and Use Committee of China Agricultural University (under the certificate of Beijing Laboratory Animal employee ID: CAU20161210-2).

Results

Evaluation of the effect of positive serum for *T. gondii* and *N. caninum*

The specificity of the mouse *T. gondii*–positive serum and mouse *N. caninum*–positive serum was detected by western blot and ELISA. Western blot results showed that the prepared *T. gondii*–positive serum reacted well with *T. gondii*–specific protein SAG1, near 25 kd, while there was no cross-reaction with *N. caninum*–positive serum. The titers of positive sera for *T. gondii* and *N. caninum* were detected by ELISA. The results showed that the titers of *T. gondii* positive sera Tg1, Tg2, Tg3, and Tg4 were 1:104,200, 1:104,200, 1: 51,200, and 1: 204,800, respectively. The titers of Nc1, Nc2, Nc3, and Nc4 in *N. caninum* positive sera were 1: 204,800, 1: 51,200, 1: 204,800, and 1: 51,200, respectively.

Identification of *T. gondii*–specific antigen by IP-MS analysis

The following results were obtained by SDS-PAGE detection: IgG antibody recognized a large number of antigenic components of *T. gondii* with good immunogenicity, and most of the recognized proteins had a molecular weight greater than 15 kd (Fig. 1). The protein collections of *T. gondii* protein lysates reactive with *T. gondii*–positive serum and *N. caninum*–positive serum were obtained by collecting SDS-PAGE strips and mass spectrometry identification. The proteins common to both protein sets were cross-reactive proteins of *T. gondii* and *N. caninum*, and only the proteins screened in the positive serum of *T. gondii* were *T. gondii*–specific proteins. A total of 662 cross-antigens of *T. gondii* and *N. caninum* and 241 specific proteins of *T. gondii* were identified by LC-MS/MS.

To better understand the components of *T. gondii*–specific antigens screened by IP-MS and their role in *T. gondii* infection, we analyzed 154 proteins (the other 87 proteins were hypothetical proteins) by GO analysis. Annotations were made based on cell composition, biological process, or molecular function. Among the identified proteins, 37% belonged to membrane proteins, 27% to cytoplasmic proteins, 16% to nucleoproteins, 7% to ribosomal-related proteins, 4% to Golgi apparatus-related proteins, 4% to endoplasmic reticulum-related proteins, 3% to cytoskeleton proteins, and the remaining 2% to mitochondrial-related proteins (Fig. 2a). They mainly play the function of signal transduction and transmembrane transport and are involved in the movement process (Fig. 2b).

Some enzymes and structural proteins that were unsuitable for diagnosis were excluded from the specific proteins of *T. gondii*. The selected amino acid sequences were compared with the *N. caninum* genome in the NCBI database (https://www.ncbi.nlm.nih.gov/). According to the level of homology, the following 19 proteins with low homology were selected as candidate proteins for bioinformatic analysis, including the existence of signal peptides, transmembrane domains, and antigenic epitopes (Table 1). Among these 19 proteins, seven proteins were selected

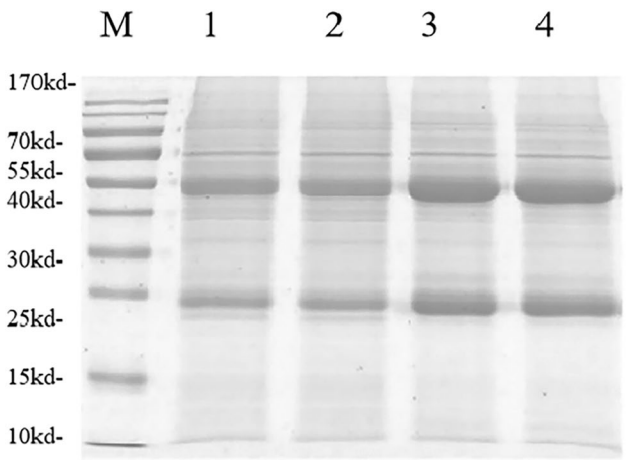

Fig. 1 The IP-enriched and purified *T. gondii* antigens were analyzed by SDA-PAGE. M: protein standard; lane: 1, 2: *T. gondii* antigen and *N. caninum* positive serum binding products; lane: 3, 4: *T. gondii* antigen and *T. gondii* positive serum binding products

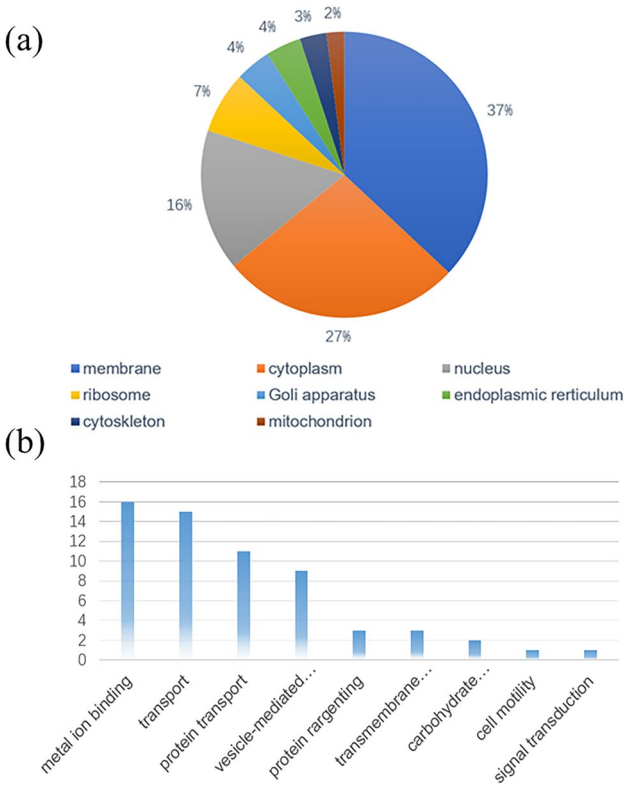

Fig. 2 Results of immunoprecipitation-mass spectrometry analysis. **a** The pie chart shows the distribution of specific antigens of *T. gondii* based on cell group classification. A total of 120 proteins have been successfully annotated. **b** The histogram shows the distribution of specific antigens of *T. gondii* based on major biological processes or molecular functional classifications. A total of 61 proteins have been successfully annotated. The ordinate represents the number of proteins

for gene cloning and prokaryotic expression, in accordance with their better solubility and antigenicity information (Table 2), which were analyzed in the ToxoDB database (http://www.toxodb.org). The seven potential proteins include four dense granule proteins and one surface antigen-related protein (SRS30A), while the other proteins are hypothetical proteins.

Expression, purification, and identification of potential proteins

PCR identification and sequencing results show that pET-28a-TGME49_247520, pET-28a-TGME49_290700, pET-28a-TGME49_204340, pET-28a-TGME49_253690, pET-28a-TGME49_237015, and pET-28a-TGME49_273130 are consistent with the ToxoDB database (Fig. 3).

The prokaryotic expression–positive plasmids containing the target gene were transformed into the expression strain Transetta, and then, prokaryotic expression and expanded culture were carried out to obtain seven recombinant proteins: rTGME49_247520-his, rTGME49_328700-his, rTGME49_290700-his, rTGME49_204340-his, rTGME49_253690-his, rTGME49_237015-his, and rTGME49_273130-his. The supernatants of uninduced bacteria, induced bacteria, lysed supernatants, and lysed inclusion bodies were collected and analyzed by SDS-PAGE electrophoresis to observe the expression of each protein. The results showed that the seven proteins were expressed in different degrees in both supernatant and inclusion bodies (Figure S1). Among them, rTGME49_237015-his and rTGME49_204340-his were highly expressed in prokaryotic cells, making them suitable for protein purification and follow-up experiments. rTGME49_237015-his and rTGME49_204340-his were purified by affinity chromatography to obtain purified recombinant proteins. The rTGME49_237015-his protein was expressed in inclusion bodies, and the ELISA pre-test results showed that the effect of inclusion body protein was poorly encapsulated and reproducible, making it unsuitable for establishing diagnostic methods. TGME49_204340 belongs to *T. gondii* dense granule protein GRA54, which is soluble expressed. The following purification results were obtained (Fig. 4). The ELISA pre-test results showed good encapsulation and reproducibility, suitable for the establishment of the diagnostic method.

Evaluation of TgGRA54 as a diagnostic antigen

The coding sequence of the 1486 bp truncated TgGRA54 gene, encoding a target protein of 495 amino acids, was inserted into the bacterial expression vector pET-28a and expressed as a his fusion protein in *E. coli* with a predicted molecular mass of 50 kDa. The rTgGRA54 was expressed mainly in the supernatant, and the expression of the recombinant protein was up to 16.6% of the total bacterial protein. The rTgGRA54 can effectively identify the *T. gondii*-positive serum and does not react with *N. caninum*-positive serum from bovine (Fig. 5). A further western blot analysis also showed that rTgGRA54 can recognize *T. gondii*-positive sera from sheep, mouse, and swine (Fig. 6a, b, c). This result indicates that the rTgGRA54 is a candidate diagnostic antigen for toxoplasmosis.

Diagnosis of *T. gondii* infection in cattle by ELISA with rTgGRA54

Seventy-two samples of *T. gondii* serum–positive and 21 samples of *T. gondii* serum–negative bovine serum were defined by IFAT and evaluated by indirect ELISA based on rTgGRA54 and rTgSAG1 antigens. The sensitivity and

Table 1 Bioinformatics of specific antigens of *T. gondii*

Gene ID	Name	Amino acid	Weight (kd)	Signal peptide	Transmembrane domain	Homology with *N. caninum*	Prokaryotic expressed
TGME49_231865	Hypothetical protein	93	10,160	No	No	0.00%	
TGME49_213050	Hypothetical protein	192	21,190	No	No	0.00%	
TGME49_275440	Dense granule protein 6	224	23,310	No	Yes	28.76%	
TGME49_286450	Dense granule protein 5	120	12,840	Yes	Yes	35.45%	
TGME49_204340	Dense granule protein 54	877	97,090	Yes	Yes	37.91%	Yes
TGME49_234640	Hypothetical protein	1625	169,460	No	No	40.36%	
TGME49_328700	Hypothetical protein	88	9610	Yes	Yes	41.54%	Yes
TGME49_254860	Hypothetical protein	1799	196,190	No	No	41.80%	
TGME49_237015	Hypothetical protein	264	29390	No	Yes	42.20%	Yes
TGME49_247520	Hypothetical protein	256	26,540	Yes	Yes	43.55%	Yes
TGME49_218240	Hypothetical protein	256	132,280	Yes	Yes	44.48%	
TGME49_225860	Hypothetical protein	1057	111,620	No	No	47.64%	
TGME49_271430	Hypothetical protein	1313	144,420	No	No	47.67%	
TGME49_291620	Hypothetical protein	1487	160,040	No	No	48.03%	
TGME49_253690	Hypothetical protein	119	12,280	No	Yes	48.46%	Yes
TGME49_233160	Hypothetical protein	1874	198,670	No	No	48.95%	
TGME49_290700	Dense granule protein 25	315	34,730	Yes	No	51.20%	Yes
TGME49_275860	Hypothetical protein	383	43,160	Yes	Yes	54.81%	
TGME49_273130	SAG1-related sequence 30A	362	38,500	No	No	55.79%	Yes

specificity of the ELISA were evaluated. Compared to IFAT, the positive coincidence rate (sensitivity) of the rTgGRA54-based ELISA was 100% and the negative coincidence rate (specificity) was 81.0%, whereas the rTgSAG1-based ELISA was 94.4% and 100%, respectively. The sensitivity of the rTgGRA54-based ELISA was better than that of the rTgSAG1-based ELISA. The rTgSAG1 (kappa = 0.886) and rTgGRA54-based ELISA tests (kappa = 0.868) are highly consistent with the IFAT test by calculating the kappa values (Table 3). The established ELISA detection method was used to detect 165 clinical bovine samples. The results showed that 134 samples were confirmed as positive by the rTgGRA54-based ELISA, and the detection rate of *T. gondii* antibody in this batch of bovine serum samples was 81.21%.

Discussion

To date, a large number of recombinant antigens have been expressed in *E. coli* and several recombinant proteins have been produced and applied for the detection of *T. gondii* infection. These proteins include rhoptry proteins, matrix proteins, microneme proteins, surface antigens, and GRAs (Kotresha and Noordin 2010). Among them, GRA proteins are considered as potential diagnostic antigens and have been used to differentiate the stages of infection.

Zhang identified 26 cross antigens via 2-DE and immunoblot analysis (Zhang et al. 2011), and a large number of cross-reactive antigens have been reported between *T. gondii* and *N. caninum* by western blot, enzyme-linked

Table 2 Bioinformatics of prokaryotic expressed proteins of *T. gondii*

Gene ID	Name	Number of epitopes	Expression level	Main protein expression pattern
TGME49_247520	Hypothetical protein	1	Low	Inclusion body
TGME49_328700	Hypothetical protein	1	Low	Both
TGME49_290700	Dense granule protein 25	1	Low	Supernatant
TGME49_204340	Dense granule protein 54	1	High	Both
TGME49_253690	Hypothetical protein	1	Low	Inclusion body
TGME49_237015	Hypothetical protein	1	High	Inclusion body
TGME49_273130	SAG-related sequence SRS30A	1	Low	Both

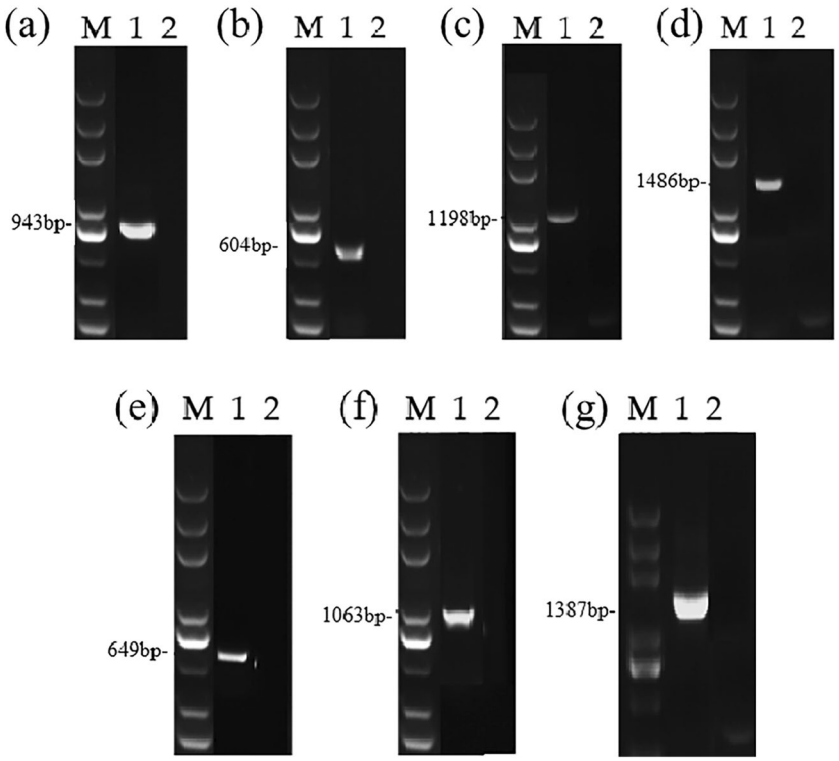

Fig. 3 PCR identification results of prokaryotic expression plasmids. **a** M: marker; lane 1, pET-28a-TGME49_247520 positive monoclonal PCR products; lane 2, negative control. **b** M: marker; lane 1, pET-28a-TGME49_328700 positive monoclonal PCR products; lane 2, negative control. **c** M: marker; lane 1, pET-28a-TGME49_290700 positive monoclonal PCR products; lane 2, negative control. **d** M: marker; lane 1, pET-28a-TGME49_204340 positive monoclonal PCR products; lane 2, negative control. **e** M: marker; lane 1, pET-28a-TGME49_253690 positive monoclonal PCR products; lane 2, negative control. **f** M: marker; lane 1, pET-28a-TGME49_237015 positive monoclonal PCR products; lane 2, negative control. **g** M: marker; lane 1, pET-28a-TGME49_273130 positive monoclonal PCR products; lane 2, negative control

immunosorbent assay, and immunohisto-chemical test (Liao et al. 2005, 2006; Zhang et al. 2007). Besides, Xue identified twenty-six cross antigens via immunoprecipitation-shotgun (Xue et al. 2016). In the study, 224 specific antigens and 662 cross anti-gens were identified by IP-MS, and the number of cross-reactive proteins identified was much higher than that of *T. gondii*–specific proteins. Among the 662 cross antigens identified by mass spectrometry, heat shock proteins (HSP90, HSP70, HSP60, HSP20, etc.), ribosomal proteins (RPSA, RPS6, RPS7, RPS25, RPS20, etc.), and some

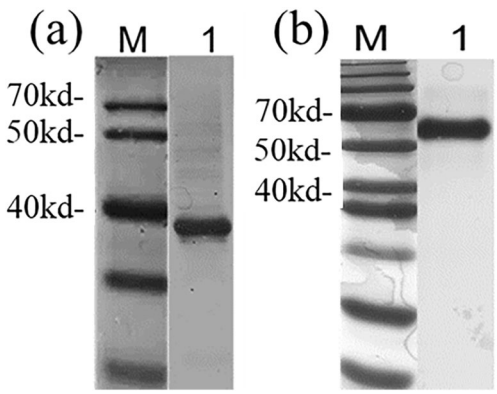

Fig. 4 SDS-PAGE identification of purified recombinant protein. **a** M: protein marker; lane 1: purified rTGME49_237015-his protein. **b** M: protein marker; lane 1: purified rTGME49_204340-his protein

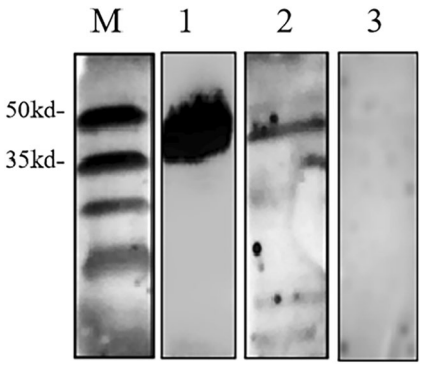

Fig. 5 Evaluation of the reactivity of rTgGRA54 with *T. gondii* positive serum from bovine. Lane 1: rTgGRA54 reacts with anti-his monoclonal antibody; lane 2: rTgGRA54 reacts with bovine *T. gondii*–positive serum; lane 3: rTgGRA54 reacts with bovine *N. canium*–positive serum

Table 3 Sensitivity and specificity of the rTgGRA54-based ELISA and rTgSAG1 protein–based ELISA using IFAT as the reference standard

IFAT	rTgGRA54-based ELISA		rTgSAG1-based ELISA		Total
	Positive	Negative	Positive	Negative	
Positive	72	4	68	0	72
Negative	0	17	4	21	21
Total	72	21	72	21	93
Sensitivity	100% (72/72)		94.44% (68/72)		——
Specificity	80.95% (17/21)		100% (21/21)		——
Agreement (κ value)	0.868		0.886		——

enzymes and structural proteins were most highly scored. GO analysis of these proteins to understand the cellular components and biological processes of the cross-antigens of *T. gondii* and *N. caninum* showed that a total of 376 proteins were successfully annotated, of which 41% were cytoplasmic proteins, 23% were membrane proteins, as well as some other organelles and skeletal proteins (Figure S2). This further proves that there is a strong similarity between *T. gondii* and *N. caninum*, which indicates the existence of great similarity proteins between *T. gondii* and *N. caninum*.

The cross-reactive antigens of *T. gondii* and *N. caninum* are important in the quest to determine common mechanisms of parasite–host interactions, but it can also affect the diagnostic specificity of serological tests. Therefore, there is an urgent need to screen for new dominant antigens with improved diagnostic abilities and to establish more sensitive and specific serological diagnostic methods.

Based on a comprehensive analysis of the transcriptional level, solubility, and immunogenicity of the seven expressed proteins, we chose the TgGRA54 protein as the basis for subsequent experimental studies. GRA54 protein of *T. gondii* is a kind of dense granule protein discovered in 2019, which regulates the formation of nymphal vacuoles and vacuolar membranes and maintains the stability of vacuoles. Cygan studied the localization of the *T. gondii* protein and found that it is localized in intracellular vacuoles and is co-located with GRA7, which was annotated (Cygan et al. 2020). At present, there are no related reports on its bioinformatics function and immunogenicity. Our study shows that the recombinant protein of *T. gondii* obtained via gene cloning, expression, and purification has good antigenicity and can be used as a candidate antigen for the diagnosis of *T. gondii*.

Compared to IFAT, the positive coincidence rate of rTgGRA54-based ELISA in our study may be lower than that of rTgSAG1-based ELISA. Moreover, our detection information reflects a substantial discrepancy in the overall serum assessment determined by either rTgSAG1 or rTgGRA54-based ELISA. The sensitivity of the rTgGRA54-based ELISA was superior to that of the rTgSAG1-based ELISA, with a considerable portion of *T. gondii*–positive serum recognizing only one antigen. The use of a single antigen in ELISA is insufficient to simultaneously examine the antibody response against the different antigens to which the host is differentially exposed depending on the stage of infection (i.e., the acute or chronic stage) (Qi et al. 2022).

Our data indicate that the ELISA test utilizing TgGRA54 has good specificity and can be used as a supplementary antigen for missed detection by TgSAG1.

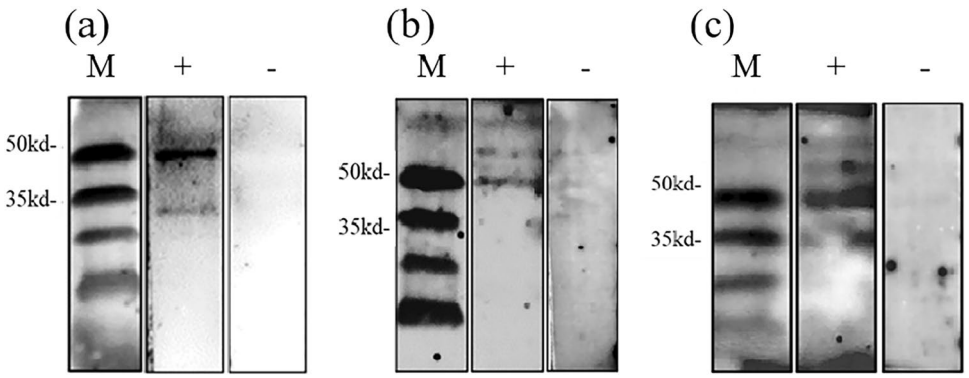

Fig. 6 Evaluation of reactivity of rTgGRA54 with *T. gondii* positive sera from other animals. **a** M: protein marker; plus sign: rTgGRA54 reacts with mouse *T. gondii*–positive serum; minus sign: rTgGRA54 reacts with mouse *T. gondii*–negative serum. **b** M: protein marker; plus sign: rTgGRA54 reacts with sheep *T. gondii*–positive serum; minus sign: rTgGRA54 reacts with sheep *T. gondii*–negative serum. **c** M: protein marker; plus sign: rTgGRA54 reacts with swine *T. gondii*–positive serum; minus sign: rTgGRA54 reacts with swine *T. gondii*–negative serum

Conclusion

In this study, a total of 241 specific antigens of *T. gondii* and 662 cross antigens between *T. gondii* and *N. caninum* were screened by IP-MS. Among them, TgGRA54 protein had high specificity and low homology with *N. caninum*. Our research shows that TgGRA54 is a reliable diagnostic marker for the serodiagnosis of *T. gondii* infection in cattle. An indirect ELISA method based on rTgGRA54 allows the highly sensitive and specific determination of antibodies against *T. gondii* in bovine serum.

Abbreviations *GRAs*: dense granule proteins; *MICs*: micronemes; *DMEM*: Dulbecco's modified Eagle's medium; *HFF*: human foreskin fibroblast; *BSA*: bovine serum albumin; *DMSO*: dimethyl sulfoxide; *CRISPR/Cas9*: clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9; *KEGG*: Kyoto Encyclopedia of Genes and Genomes; *GO*: Gene Ontology; *IP-MS*: Immunoprecipitation-Mass Spectrometry; *WB*: western blot

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Author contribution J-L conceived the project. Z-XF and H-FL performed the experiments and drafted the manuscript. JL, QL, and L-FW participated in the design of the study and helped to draft the manuscript. Z-PS, X-MW, Y-QP, and Z-FZ participated in the interpretation of the data. All authors read and approved the final manuscript.

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Data availability Data supporting the conclusions of this article are included within the article and its additional files.

Declarations

Ethics approval The experiments were performed in strict according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. All experimental animal procedures and protocols were approved by the Institutional Animal Care and Use Committee of China Agricultural University.

Consent to participate Done and confirmed.

Consent for publication Done and confirmed.

Competing interests The authors declare no competing interests.

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