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DEVELOPMENT AND APPLICATION OF AN INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY USING RECOMBINANT MAG1 FOR SERODIAGNOSIS OF *TOXOPLASMA GONDII* IN DOGS

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ABSTRACT: Serologic tests are widely accepted and applied as means to detect anti-*Toxoplasma gondii* immunoglobulin G antibodies. In this study, recombinant matrix antigen (rMAG1) was induced by isopropyl- β -D-thiogalactoside and purified by nickel–nitrilotriacetic acid purification system. We then developed and optimized an indirect enzyme-linked immunosorbent assay (ELISA) through checkerboard assays using serial dilutions of antigens and sera to assess the potential use of rMAG1 in serologic detection of *T. gondii* infection in dogs. Serum samples from 93 domestic dogs were analyzed by western blot and rMAG1-ELISA. The results were compared with those obtained from an ELISA with the soluble *Toxoplasma* lysate antigens (TLA). We found that although yielding an excellent agreement (96.7%) with western blot data ($\kappa = 0.9659$), rMAG1-ELISA produced higher sensitivity (93.9% vs. 87.8%) and specificity (98.3% vs. 96.7%) than TLA-ELISA. In addition, receiver operating characteristic analysis also revealed that rMAG1-ELISA is in more agreement with western blot (area under the curve [AUC] = 0.985) relative to TLA-ELISA (AUC = 0.955). These results indicated that the rMAG1-ELISA established in this study provides a promising and reliable tool for serologic detection of *T. gondii* infection in dogs.

Toxoplasma gondii is an obligate intracellular protozoan parasite that has a worldwide distribution and can infect almost all warm-blooded animals, including humans and dogs (Dubey, 2004; Innes, 2010). Toxoplasmosis caused by *T. gondii* is a lethal threat to immunocompromised individuals (Tenter et al., 2000; Dubey et al., 2012). People can become infected with *T. gondii* via ingestion of undercooked or raw meat containing tissue cysts or ingestion of oocysts from contaminated food or water (Pittman and Knoll, 2015). Dogs infected with *T. gondii* pose a potential threat and health risk to consumers because some of them are treated as food animals in China. Thus, it is of great importance to evaluate the role of domestic dogs in *T. gondii* transmission.

Serologic tests have been widely applied and accepted for *T. gondii* infection diagnosis on the basis of the detection of immunoglobulin (Ig)G antibodies in humans and animals (Kotresha and Noordin, 2010; Holec-Gasior, 2013). Among the various existing serologic methods, the enzyme-linked immunosorbent assay (ELISA) is simple, rapid, and economical, rendering it suitable for clinical diagnosis applications. Compared with other detection methods such as polymerase chain reaction (PCR) and indirect fluorescence antibody test, which require special equipment, ELISA is much cheaper and easier. Recombinant products, such as MIC2, MIC3, M2AP, GRA3, GRA7, and SAG1 gene products, had been used as effective tools in distinguishing *T. gondii*-infected individuals from *T. gondii*-uninfected individuals performed by ELISAs (Beghetto et al., 2006). Furthermore, ELISA methods had been established to determine different Ig antibodies (IgG, IgM, and IgA) against *T. gondii* (Montoya, 2002).

The matrix antigen MAG1 is a 65-kDa protein abundantly expressed within the cyst and in the cyst wall of *T. gondii* (Parmley et al., 1994). Ferguson and Parmley (2002) reported that the

protein is very immunogenic and is expressed during both tachyzoite and bradyzoite development. High levels of IgG antibody titers against MAG1 were found in *T. gondii*-infected humans and pigs (Gamble et al., 2000; Di Cristina et al., 2004). Among the several recombinant proteins of *T. gondii*, MAG1 has been demonstrated to be a good candidate for the serodiagnosis of toxoplasmosis (Holec et al., 2007; Holec-Gasior et al., 2012). In this study, the recombinant MAG1 (rMAG1) protein was evaluated as a potential diagnostic marker of *T. gondii* infection in dogs by an indirect ELISA method. In the meantime, a traditional ELISA method based on soluble *Toxoplasma* lysate antigens (TLA) was established. In addition, we compared the performance of the MAG1-ELISA against TLA-ELISA for *T. gondii* detection in dog serum samples.

MATERIALS AND METHODS

Extraction of *T. gondii* genomic DNA

Tachyzoites of *T. gondii* RH strain were intraperitoneally inoculated into BALB/c mice and peritoneal fluid was harvested after 3–5 days. Parasites were washed twice in phosphate-buffered saline (PBS) and centrifuged at 1,000 g for 5 min as previously described (McLeod et al., 1984). The genomic DNA of *T. gondii* was then extracted using a universal genomic DNA extraction kit (TaKaRa, Shanghai, China) according to the manufacturer's instructions.

BALB/c mice used as experimental animals were treated according to the recommendations in the Guide for the Regulation for the Administration of Affairs concerning Experimental Animals of the People's Republic of China. Animal experiments were approved by Zhejiang University Experimental Animal Ethics Committee (permit number: ZJU201308-1-10-072). In this experiment, 5 female BALB/c mice were used to harvest tachyzoites of *T. gondii* to obtain TLA and genomic DNA. The collection of serum samples from dogs in the present study was performed according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

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Preparation of rMAG1 and TLA

Genomic DNA of *T. gondii* was extracted from about 10⁷ tachyzoites (RH strain maintained in Vero cells) using a universal genomic DNA extraction kit (TaKaRa) according to the manufacturer's instructions. On the basis of MAG1 sequence (GenBank accession number AF251813), PCR primers spanning a 529-base pair amplification product (sense: 5'-CGGGGTACCGTGCCA-GAGCTACCAGAAAGTGG-3'; antisense: 5'-CGCGAATTCCGC-CAGATCCCTGAACCCTTAG-3') were designed with the inclusion of *Kpn*I and *Eco*RI restriction sites (underlined), respectively. Amplification reaction was performed at 95 °C for 5 min, followed by 30 cycles of 94 °C for 45 sec, primer annealing at 63 °C for 45 sec, extension at 72 °C for 45 sec, and a final extension at 72 °C for 8 min. The amplified product was purified and cloned into the *Kpn*I/*Eco*RI sites of the expression vector pET-28b (Novagen, Shanghai, China) to generate the recombinant plasmid pET28-MAG1. Upon restriction enzyme digestion and sequencing, the right clone was introduced into *Escherichia coli* BL21 (DE3) cells (Novagen) and gene expression was carried out according to the standard procedures. The rMAG1 protein was purified by affinity chromatography using a nickel-nitrilotriacetic acid (Ni-NTA) agarose column system (Qiagen, Shanghai, China) according to the manufacturer's protocol.

The recombinant protein (10 µg/lane) was analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% gel. Western blot was then carried out to determine the reactivity of the antibody against *T. gondii*-positive sera of mice at a 1:200 dilution, followed by anti-mouse IgG–alkaline phosphatase conjugate (dilution, 1:5,000) (Sigma-Aldrich, Shanghai, China).

Purified *T. gondii* tachyzoites of RH strain were used to extract TLA, which was diluted to a final concentration of 1 mg/ml in PBS as described previously (Zhu et al., 2008).

Serum samples

Dog serum samples were collected from the affiliated animal hospital of Zhejiang University. Blood samples of these dogs (65 were domestic and 28 were homeless, and age distributions were as follows: 21 were <1 yr old, 47 were 1–3 yr old, 25 were >3 yr old) were collected from February to May 2014 in Hangzhou, China. Sera were separated by centrifugation at 1,500 g for 10 min and kept at –20 °C until use. Dog serum samples were tested by western blot 3 times as described elsewhere (Pardini et al., 2012) and positive and negative serum controls were included throughout the experiment. Briefly, TLA proteins were run in SDS-PAGE with a 12% gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Shanghai, China); after blocking with 5% milk–PBS containing 0.1% Tween-20, the membranes were subsequently incubated with sera diluted 1:100 in blocking solution, followed by rabbit anti-canine IgG–horseradish peroxidase conjugate (HRP) antibodies (dilution, 1:3,000) (Sigma-Aldrich). Finally, the membranes were tested by enhanced chemiluminescence (Thermo-Scientific, Shanghai, China).

ELISA

An indirect ELISA was carried out and optimized with serum samples. Briefly, 96-well microplates were coated overnight at 4 °C with purified rMAG1 or TLA diluted in 100 mM carbonate–

bicarbonate buffer (pH 9.6). The wells were washed 3 times and blocked with 5% skim milk in 0.01 M PBS containing 0.1% Tween-20 (PBST) for 1 hr at 37 °C. After 3 washes with PBST, 100 µl of serum samples from canines diluted in PBST–5% milk was added to each well and incubated for 1 hr at 37 °C. After washing, the reaction mixture was incubated with rabbit anti-canine IgG–HRP conjugate antibodies (dilution, 1:1,500). After incubation for 1 hr and subsequent washing, color was developed by the addition of 100 µl of substrate solution consisting of tetramethylbenzidine chromogenic substrate (Sigma-Aldrich), and stopped by adding 50 µl of 2 M H₂SO₄. The optical densities (ODs) were measured at 450 nm in a microplate reader (Chromate 4300; Awareness Technology Inc., Palm City, Florida). Each serum sample had three parallel wells and each well was read twice and the cutoff point of a positive sample was set to be at least twice of the negative sample at any dilution (Zhu et al., 2008).

The specificity of the rMAG1-ELISA was examined by using the positive canine serum samples of canine parvovirus (CPV), *T. gondii*, *Neospora caninum*, *E. coli*, *Salmonella enterica*, and *Listeria monocytogenes* provided by the affiliated animal hospital of Zhejiang University. Four of each kind of positive serum sample were diluted 1:200 by PBST–5% milk and healthy canine serum was added as negative control. Data were measured at 450 nm in a microplate reader as described above.

Statistical analysis

Statistical analysis was performed using SPSS software, ver. 18 (SPSS Inc., Chicago, Illinois). Relative sensitivity and specificity of the ELISAs were determined by receiver operating characteristic (ROC) analysis (Swets, 1988), and agreement among serologic tests was calculated and evaluated with kappa values (Conraths and Schares, 2006).

RESULTS

Expression and purification of rMAG1

The MAG1 encoding gene was amplified by PCR from genomic DNA of *T. gondii* RH strain and cloned into expression vector pET-28b to construct recombinant plasmid pET28-MAG1 with a hexahistidine tail sequence for subsequent purification. The MAG1 antigen was expressed in *E. coli* BL21 (DE3) bacterial strain. As shown in Figure 1A, a band of approximately 42 kDa was induced by isopropyl-β-D-thiogalactoside (IPTG) and highest expression level was reached at 4 hr. rMAG1 was purified using a Ni-NTA agarose column system (Fig. 1B) and the immunoreactivity of the expressed protein was confirmed by western blot using anti-His-tag primary antibodies and the dog serum positive for anti-*T. gondii* IgG antibodies, respectively (Fig. 1C).

Detection of canine serum samples by western blot

Of a total of 93 serum samples collected from domestic dogs, 33 (35.48%) and 60 (64.52%) samples were shown to be positive and negative respectively for anti-*T. gondii* IgG antibodies on western blots (see Suppl. Fig. S1).

Development of ELISA on the basis of TLA and rMAG1

Two separate IgG ELISAs were developed using the rMAG1 and TLA as coating antigens. The optimal working concentra-

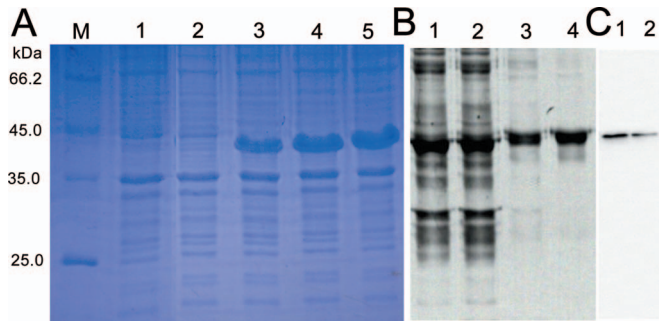


FIGURE 1. Expression and identification of recombinant MAG1 of *Toxoplasma gondii*. (A) Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of MAG1 expressed in *Escherichia coli* using 12% acrylamide gel and colored by Coomassie blue. Lane 1: Induced control culture of cells with empty vector; lane 2: induced control culture of cells with expression vector and without IPTG; lanes 3–5: expression of MAG1 after 2, 4, and 6 hr of induction, respectively. (B) SDS-PAGE analysis of purified rMAG1. Lanes 1, 2: rMAG1 protein before purification; lanes 3, 4: rMAG1 was purified with 100 mM and 200 mM imidazole, respectively. (C) Western blot detection of rMAG1 by anti-His-tag primary antibodies (lane 1) and dog serum positive for anti-*T. gondii* IgG antibodies (lane 2). Color version available online.

tions were shown to be 15 $\mu\text{g/ml}$ for rMAG1 and 20 $\mu\text{g/ml}$ for TLA (Fig. 2A, B), which were determined by checkerboard assays using serial dilutions of antigens and sera based on the principle that OD ratio of the positive to the negative samples should be above 2.1; thus the cutoff values were determined to be 0.348 (\bar{x} = 0.141, SD = 0.069) for rMAG1 and 0.369 (\bar{x} = 0.165, SD = 0.068) for TLA. The appropriate dilutions of sera were confirmed to be

1:200 for both rMAG1 and TLA when the OD values were around 0.5 (Fig. 2C, D).

Positive canine serum samples of CPV, *T. gondii*, *N. caninum*, *E. coli*, *S. enterica*, and *L. monocytogenes* were used to evaluate the specificity of the established rMAG1-ELISA assay. Our results (Fig. 3) indicated that only the *T. gondii*-positive serum could be recognized by rMAG1 protein, indicating that there are no cross-reactions and suggesting that the established assay could provide accurate detection.

Clinical application of the TLA-ELISA and rMAG1-ELISA

A total of 93 dog serum samples was tested for the presence of anti-*T. gondii* IgG antibodies by TLA-ELISA and rMAG1-ELISA, respectively. From our results, there were 31 and 62 samples that tested positive and negative respectively by TLA-ELISA, whereas 32 and 61 samples tested positive and negative respectively by rMAG1-ELISA, as shown in Table I. Compared with western blot data, 1 false-positive and 2 false-negative samples were found in rMAG1-ELISA, whereas 2 false-positive and 4 false-negative samples were detected in TLA-ELISA (Table I). These results suggested that rMAG1-ELISA presents a higher relative sensitivity (93.9%, 31/33) and relative specificity (98.3%, 59/60) than TLA-ELISA, with a relative sensitivity of 87.8% (29/33) and relative specificity of 96.7% (58/60). Both the agreements of TLA-ELISA (κ = 0.9320) and rMAG1-ELISA (κ = 0.9659) fell into the category of near-perfect agreement (κ > 0.81).

ROC analysis revealed an area under curve (AUC) of 0.955 ± 0.24 (95% confidence interval [CI] 0.904 to 0.997) for TLA-ELISA and 0.985 ± 0.014 (95% CI 0.912 to 1.000) for rMAG1-ELISA, respectively (Fig. 4).

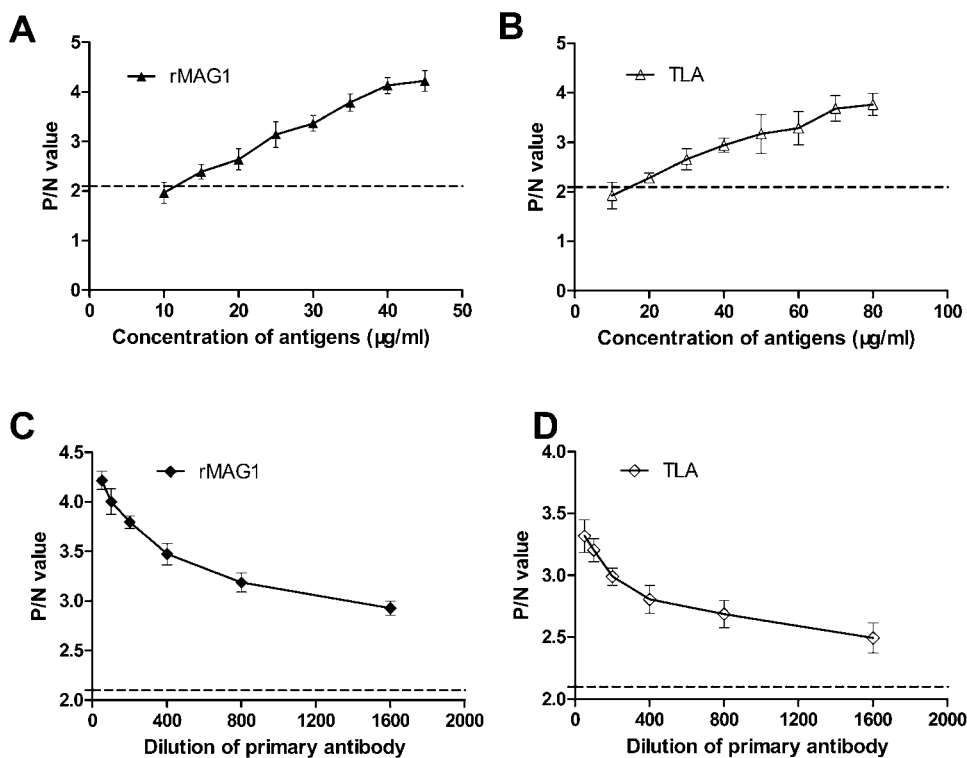


FIGURE 2. Optical density ratios of the positive to the negative samples (P/N) detected by rMAG1-ELISA (A) and TLA-ELISA (B) using coating antigen of different concentrations. The optical P/N values were detected by rMAG1-ELISA (C) and TLA-ELISA (D) using different dilutions of primary antibodies. The cutoff value is indicated by a horizontal line.

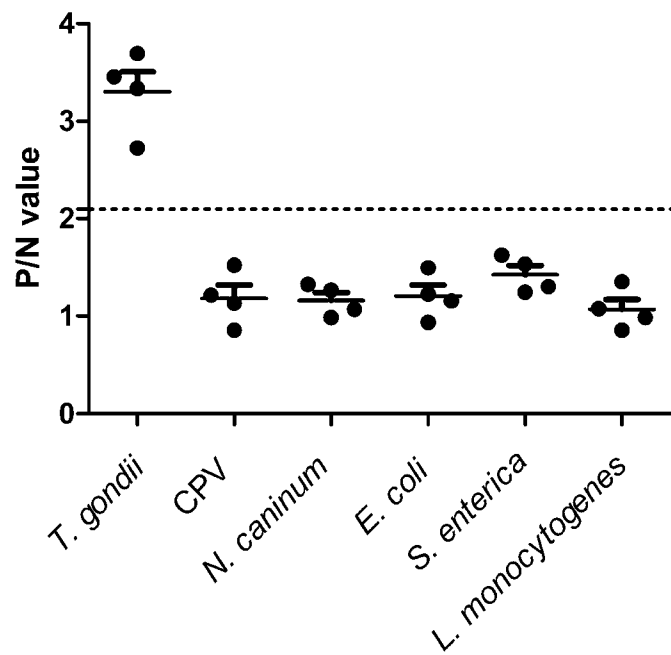


FIGURE 3. Optical density ratios of the positive to the negative samples (P/N) detected by rMAG1-ELISA using positive serum samples of *Toxoplasma gondii*, canine parvovirus, *Neospora caninum*, *Escherichia coli*, *Salmonella enterica*, and *Listeria monocytogenes*. The cutoff value is indicated by a horizontal line.

DISCUSSION

The food-borne apicomplexan protozoan *T. gondii* is found worldwide and is potentially capable of infecting almost any nucleated cell type with an astonishingly broad host range across humans, birds, livestock, and other warm-blooded animals (Jones and Dubey, 2012). Thus, it is necessary to accurately detect *T. gondii* infection for better management and proper control of *Toxoplasma*. Various ELISAs based on recombinant proteins have been developed and applied for the serodiagnosis of *T. gondii* infection in humans and animals. Beghetto et al. (2006) reported that recombinant antigens could provide the basis for standardized commercial tests for the serodiagnosis of toxoplasmosis. Other recombinant proteins had been used for serodiagnosis, such as SAG1 (Chahed Bel-Ochi et al., 2013), ROP2 (Liu et al., 2012), MIC1 (Holec et al., 2008), and GRA7 (Wang et al., 2014). Antibodies against *T. gondii* produced in chronic and acute infection could be recognized by MAG1 protein, but it is mostly associated with chronic toxoplasmosis since MAG1 is abundantly expressed within the cyst and in the cyst wall surrounding the bradyzoites (Ferguson and Parmley, 2002); thus there may be false-negative results when applied by MAG1-based serology methods from acute toxoplasmosis samples since lower anti-MAG1 antibodies would be produced in this stage. MAG1 has been reported to induce a strong immune response in humans and animals (Xiao et al., 2013). These antigenic properties of MAG1 make it a promising serologic marker for *T. gondii* detection.

In the present study, we utilized the *E. coli* expression and Ni-NTA agarose column purification systems to obtain abundant rMAG1 antigen of *T. gondii*. ELISAs based on rMAG1 and TLA were developed and optimized by checkerboard assays and cutoff values were determined to be 0.348 ($\bar{x} = 0.141$, $SD = 0.069$) for

TABLE I. Detection of *Toxoplasma gondii* infection in dogs by TLA-ELISA and rMAG1-ELISA in comparison with western blot.

Detection method	Western blot		Total
	Positive	Negative	
TLA-ELISA			
Positive	29	2	31
Negative	4	58	62
Total	33	60	93
rMAG1-ELISA			
Positive	31	1	32
Negative	2	59	61
Total	33	60	93

rMAG1 and 0.369 ($\bar{x} = 0.165$, $SD = 0.068$) for TLA. No cross-reactions were found when applied with positive serum samples of CPV, *N. caninum*, *E. coli*, *S. enterica*, and *L. monocytogenes*, suggesting that the optimized rMAG1-ELISA assay is specific for *T. gondii*-positive serum. Upon testing on dog serum samples, of which infection status was first determined by western blot, 1 false-positive and 2 false-negative samples were found in the rMAG1-ELISA system, whereas the TLA-ELISA system yielded 2 false-positive and 4 false-negative samples. Higher sensitivity (93.9%) and specificity (98.3%) were detected by rMAG1-ELISA than by TLA-ELISA, with a sensitivity of 87.8% and a specificity of 96.7%. ROC analysis also revealed that rMAG1-ELISA yields a relatively higher accuracy ($AUC = 0.985$) compared with TLA-ELISA ($AUC = 0.955$). ELISAs based on SAG1 (Jalallou et al., 2012) and ROP2 (Capobiango et al., 2015) are excellent tools for diagnosis of acute toxoplasmosis, whereas rMAG1-ELISA is more suitable for chronic toxoplasmosis. Thus, the combination of rMAG1-ELISA and other detection methods could be used as an alternative tool for the determination of chronic and acute

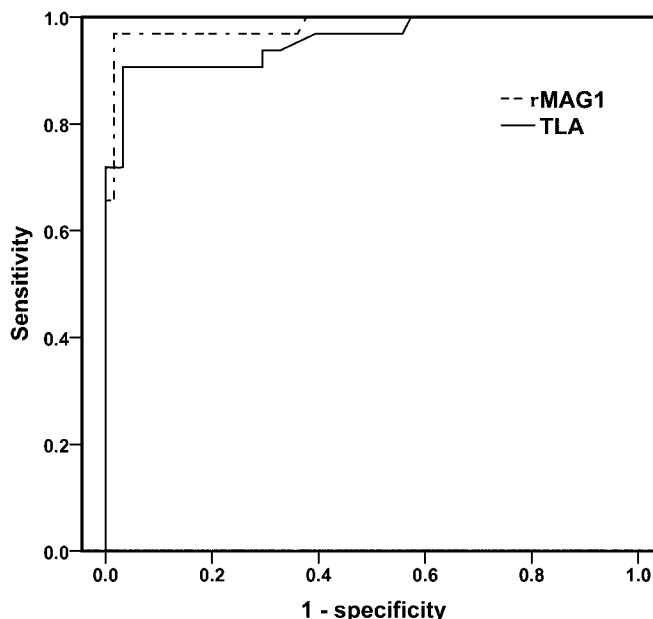


FIGURE 4. Receiver operating characteristics (ROC) analysis of rMAG1-ELISA and TLA-ELISA applied to positive vs. negative individuals identified by western blot.

toxoplasmoses. Most important, our preliminary data pointed out the prevalence of toxoplasmosis in domestic dogs in Hangzhou, Zhejiang Province, China. In view of that, a large-scale epidemiological investigation should follow up to control toxoplasmosis in a way to ensure public health.

According to the findings in this study, the ELISA based on affinity-purified rMAG1 protein of *T. gondii* was useful for the detection of antibodies to this protozoan parasite in naturally infected dogs with relatively high specificity and sensitivity.

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