Title: Evaluation of recombinant antigens in combination and single formula for diagnosis of feline toxoplasmosis

Running title: Diagnosis of *Toxoplasma* infection in cats

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Abstract

Cats are the only definitive hosts of *Toxoplasma gondii* and constitute an essential source of infection to all warm blooded animals and humans. Diagnosis of *T. gondii* infection in cats is fundamental for proper management and control of infection in humans and animals. In the current study, we have evaluated the diagnostic performance of tachyzoite lysate antigen (TLA) and different *T. gondii* recombinant antigens including surface antigen 2 (SAG2), dense granule proteins 2, 6, 7, 15 (GRA2, GRA6, GRA7, GRA15) and microneme 10 protein (MIC10) in immunoglobulin G enzyme linked-immunosorbent assay (IgG ELISA) using cat serum samples, with reference to latex agglutination test (LAT). Remarkably, TLA showed better performance than other recombinant antigens in IgG ELISAs as compared to LAT, with concordance and Kappa values of 94.27% and 0.93, respectively. Furthermore, to improve the reactivity of the recombinant antigens, we have developed IgG ELISAs using different combinations with these recombinant antigens. Strikingly, a combination of SAG2 and GRAs has relatively similar performance as TLA evidenced by concordance and Kappa values of 94.27% and 0.81, respectively. The developed ELISA with a combination of recombinant antigens can be used as a promising diagnostic tool for routine testing of *T. gondii* infection and mass screening in cats. The major advantages of this assay are the high sensitivity and specificity, lower cost, safer production and easiness of standardization in various laboratories worldwide.

Keywords: ELISA; Recombinant proteins; Serodiagnosis; Toxoplasmosis

1. Introduction

Toxoplasmosis is globally prevalent zoonotic disease caused by the intracellular protozoan parasite *Toxoplasma gondii* (*T. gondii*) (Tenter et al., 2000). Prevalence of infection is often highest in regions of the world that have hot, humid climates and lower altitudes. Felids are the definitive hosts of this parasite and mostly all warm-blooded mammals including humans serve as intermediate hosts (Dabritz and Conrad, 2010; Elmore et al., 2010). Transmission to humans and animals occurs via ingestion of food and water contaminated with infectious oocysts shed in cat feces (Dubey and Beattie, 1988). Postnatal infection causes fatal encephalitis in immunocompromised patients such as those with AIDS. Congenital infection may cause abortion, neonatal deaths, or foetal abnormalities result in blindness and mental retardation in pregnant woman (Jones et al., 2003; Luft et al., 1984). In animals, it causes abortion and stillbirth in all types of livestock, especially in sheep and goats resulting in significant reproductive and economic losses (Buxton, 1998).

Little attention has been paid to feline toxoplasmosis, because *T. gondii* infection in cats is usually asymptomatic and latent infections are the most common. Development of accurate diagnostic tests in cats is crucial for proper management and control of *T. gondii* infection. Several assays have been developed for diagnosis of *T. gondii* infection in humans and animals. Serological techniques seem to be the most suitable for routine mass screening of samples (Montoya, 2002). TLA has been traditionally used in serological detection of *T. gondii* infection, however, the recombinant proteins offer better test standardization with less production costs. Although number of immunodominant antigens has been used for serodiagnosis of feline toxoplasmosis (Cai et al., 2015; Hosseininejad, 2012; Huang et al., 2002, Kimbita et al. 2001),

61 none of these showed all criteria required to replace the native antigen of *T. gondii* in serological
62 tests. Therefore, further research is required to develop an accurate serodiagnostic test in cats. In
63 the present study, we have evaluated the diagnostic performance of recombinant protein antigens
64 and different combinations for the serodiagnosis of feline toxoplasmosis. Our results indicate
65 that a combination of SAG2, GRA2, GRA6, GRA7, and GRA15 can be used as a promising tool
66 for detection of *T. gondii* infection in cats using ELISA. This is the first report demonstrating the
67 usefulness of combination formula antigen in diagnosis of feline toxoplasmosis.

68

2. Materials and Methods

2.1. Preparation of T. gondii lysate antigen (TLA)

Tachyzoites were maintained in human foreskin fibroblast (HFF) cells cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Grand Island, NE, U.S.A.) supplemented with 7.5% heat-inactivated fetal bovine serum (FBS). TLA preparation was based on the standard procedure, by three times freezing and thawing of tachyzoites obtained from *T. gondii* (ME49 strains).

2.2. Cloning of T. gondii genes

The cloning of *T. gondii* genes encoding SAG2 (from position 79 to 516 bp), GRA2 (from position 1 to 558 bp), GRA6 (from position 1 to 690 bp), GRA7 (from position 91 to 711 bp), GRA15 (from position 1 to 1650 bp), MIC10 (from Position 148 to 597 bp) proteins was conducted. The RNA was extracted from the purified tachyzoites (RH strain) using a commercial RNeasy mini kit (QIAGEN) and reverse transcribed using One step RNA PCR kit for reverse transcription (One step RNA PCR kit, Takara, Japan) and then used as a template to amplify the target genes. Oligonucleotide primers used for amplification of *T. gondii* genes were shown in Table 1. The amplified cDNAs of *SAG2*, *GRA6*, and *GRA7* were double digested with *Bam*HI and *Eco*RI whereas that of *GRA2*, *GRA15* and *MIC10* were double digested with *Bam*HI and *Xho*I, and subcloned into the identical restriction sites of pGEX-5X-1 or pGEX-6P2 (GE Healthcare UK Ltd.). Plasmids were transformed into *Escherichia coli* DH5α competent cells. Cycle sequencing reactions were carried out using a BigDye Terminator Cycle Sequencing kit

89 Ver. 3.1 according to the manufacturer's protocol (Applied Biosystems, USA), and each sample
90 was analyzed using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

91 *2.3. Expression and purification of recombinant fusion proteins*

92 The resulting plasmids were transfected in *E. coli* strain BL21 (DE3) pLysS cells. Then,
93 the GST-fused proteins were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a
94 final concentration of 1 mM with mild shaking at 23ºC overnight. The cells were centrifuged at
95 5,000 × *g* for 20 min and the bacterial pellet was resuspended with 20 ml pre-chilled STE buffer
96 (150 mM NaCl, 50 mM Tris-HCl [pH 9.5] and 1 mM EDTA [pH 8.0]), then stored at -20ºC.
97 After thawing, the cells were disrupted by sonication on ice for 10 min, and 20% (w/v) Triton X-
98 100 in 1 × PBS was added to the samples to be a final concentration of 1% (w/v) Triton X-100.
99 Total proteins in the soluble fraction were affinity purified by glutathione-Sepharose beads
100 according to the manufacturer's protocols (Pharmacia biotech, Uppsala, Sweden). The eluted
101 fractions were dialysed against PBS and the amount of recombinant protein was measured using
102 both SDS-PAGE and the Coomassie protein assay reagent kit using BSA as a calibration
103 standard according to the manufacturer's protocol (Pierce Biotechnology, Inc., USA). All
104 recombinant proteins remained soluble during purification and conservation. Apparent
105 recombinant antigens on SDS-PAGE match the expected size of each protein (data not shown).

106 *2.4. Cat serum samples*

107 A total of 419 serum samples were obtained from cats visiting animal hospitals in
108 Tokachi prefecture. The serum samples were then prepared and stored at −30°C for future use.

109 *2.5. IgG ELISA*

MaxiSorp plates (Nunc, Denmark) were coated overnight at 4°C with either single recombinant antigen, mixture of recombinant proteins, or TLA at a final concentration of 1 µg/ml of each antigen in a coating buffer (50 mM carbonate, pH 9.6) and were incubated. Plates were washed and blocked with 3% skim milk in phosphate buffer saline (PBS-SM) for 1 hr at room temperature (RT). 50 µl cat sera diluted at 1:200 were added to the wells in duplicate and incubated for 1 hr at RT. After washing, the plates were incubated with horseradish peroxidase (HRP)-conjugated goat anti-cat IgG (GE Healthcare UK Limited, Buckinghamshire, U.K.) diluted at 1:4,000 with PBS-SM at RT for 1 hr. Thereafter, plates were washed 6 times before the 100 µl substrate solution [0.1 M citrate buffer, pH 4, 0.003% H2O2 and 0.3 mg/m*l* 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); Sigma-Aldrich] was added to each well. The absorbance was measured at 415 nm after 30 min of incubation at RT using an ELISA reader (Corona microplate reader MTP-120; Corona, Tokyo, Japan). Each serum sample was examined twice. The results were determined for each sample by calculating the mean optical density (OD) reading of duplicate wells. The cutoff value of each single recombinant antigen, mixture of recombinant proteins, or TLA antigen was calculated as the average OD plus 3 standard deviations of 10 cat serum samples, seronegative by LAT, western blot and immunofluorescence tests.

2.6. Latex agglutination test (LAT)

The LAT was performed according to the kit manufacturer's instructions (Toxocheck-MT, Eiken Chemical, Tokyo, Japan). Samples were considered positive when agglutination was observed at a dilution of 1:32 or greater.

2.7. Statistical analysis

The results of LAT and ELISA were estimated by the percentage of agreement, the sensitivity and specificity, and the kappa values with 95% confidence interval (http://vassarstats.net/). The strength of agreement was graded with kappa values of fair (0.21 to 0.40), moderate (0.41 to 0.60), substantial (0.61 to 0.80), and perfect (0.81 to 1.0).

3. Results

Cat serum samples were tested primarily by LAT as a reference test. Out of 419 cat samples, 73 were positive and 346 were negative. The sensitivity, specificity, concordance and kappa values obtained with the use of different recombinant antigens and TLA were variable (Table 2). Among these antigens, TLA yielded a perfect concordance with LAT results, as evidenced by kappa values of (0.93) with a high sensitivity and specificity 97.29% and 93.62%, respectively. Recombinant SAG2 and GRA6 proteins showed greater performance than other recombinant proteins with substantial kappa values of 0.67 and 0.62, respectively. On the other hand, recombinant GRA2 and GRA7, GRA15 and MIC10 proteins exhibited lesser performance in detection of *T. gondii* antibodies. These results indicate that TLA has a better performance than single recombinant proteins in diagnosing feline toxoplasmosis.

To further improve the diagnostic performance of IgG ELISA, different combination formula of recombinant proteins (M1: GRA6+GRA7; M2: GRA2+GRA7; M3: SAG2+GRA7; M4: SAG2+GRA6; M5: GRA2+GRA6+GRA7+GRA15; M6: SAG2+GRA2+GRA6+GRA7+GRA15) was used as ELISA antigen. Results were compared to these of LAT to assess their specificity and sensitivity. The M1 and M5 had a moderate concordance as evidenced by kappa values of 0.58 and 0.5, respectively. Fair concordance was yielded by M2 with kappa value of 0.35. Substantial concordance was yielded by M4 and M3 with kappa values of 0.72 and 0.62, respectively. Noteworthy, M6 exhibited the highest concordance, as evidenced by kappa value of 0.81, sensitivity, 89.19% and specificity 95.36 % (Table 2). Indeed, the reactivity of IgG ELISA for M6 was relatively similar to TLA-ELISA as compared to the results of LAT (Fig. 1). Collectively, IgG ELISA based on combination formula

of recombinant antigens has more potent diagnostic performance than this based on single recombinant antigen for detection of *T. gondii* infection in cats.

4. Discussion

Development of an accurate diagnostic method to detect *T. gondii* in cats is required to minimize the risk of transmission to humans and animals. Although LAT offers the advantage that can be used in serum samples from various species including felines, it does not provide information on the stage of infection as it did not detect adequately *T. gondii*-specific IgM in feline serum (Lappin and Powell, 1991). Recently, it was reported a LAT based on an acute phase recombinant protein in order to discriminate acute from chronic infection in humans, although it could also be applied in cats (Peretti et al., 2016). Bacterial recombinant antigens offer many advantages in the diagnosis as they allow better standardization of the tests and reduce the costs of production and purification (Pietkiewicz et al., 2004). However, many recombinant proteins have shown poor antigenicity and reactivity with field samples when they tested as ELISA antigen. Alternative approach is the use of antigens combination formula representative of the whole complex of *T. gondii* antigens.

A limited number of studies evaluated IgG ELISA assays based on recombinant antigens to diagnose feline toxoplasmosis in single but not in combination formula (Cai et al., 2015; Hosseininejad, 2012; Huang et al., 2002, Kimbita et al. 2001). On the other hand, several recombinant combinations were employed for the detection of *T. gondii* using IgG ELISA in human sera. For instance, the combination of recombinant GRA7 with ROP2 has increased the sensitivity to 96% (Jacobs et al., 1999), while combination of GRA7, GRA8, and SAG1 resulted in an improved sensitivity and specificity of the assay (Aubert et al., 2000). Likewise, GRA1 and GRA6 showed 98% sensitivity (Lecordier et al. 2000), and combined GRA7, GRA8, SAG2, and H4 has a sensitivity and specificity of 90% and 97%, respectively (Li et al., 2000). Combination

of SAG1, GRA1, and GRA7 resulted in 100% sensitivity (Pietkiewicz et al., 2004). Moreover, combined GRA8, GRA6 with SAG2 has exhibited 94.4% sensitivity (Holec et al., 2008). Combination of SAG1 and GRA5 mixed with MAG1 or GRA2 or ROP1 increased sensitivity to 92.6%, 93.1% and 94.2%, respectively (Holec-Gąsior and Kur, 2010).

In the present study, the diagnostic utility of IgG ELISAs employing TLA, six recombinant antigens and six cocktail formula was evaluated in cat sera for detection of *T. gondii* infection. The IgG ELISAs with single recombinant protein formula showed weaker performance than that with TLA. Recombinant SAG2 protein has a greater performance than other recombinant proteins. This finding was consistent with previous investigators (Huang et al., 2002) who reported the usefulness of recombinant SAG2 in diagnosis of feline toxoplasmosis. Different levels of sensitivity and specificity of recombinant GRA7 between our results and those of previous study (Cai et al., 2015) could be attributed to variations in cloning strategies and recombinant protein purification methods (Kotresha and Noordin, 2010, Holec-Gąsior, 2013). Interestingly, combination formula composed of SAG2, GRA2+GRA6+GRA7+GRA15 exhibited relatively similar performance as TLA ELISA. Our results seemed to be consistent with previous studies noted that combination antigen formula improves the diagnostic performance of an immunoassay. The greater diagnostic performance of combination antigen formula may be due to recognition of multiple different epitopes of various antigens by specific antibodies present in the serum from acute and/or chronic *T. gondii* infection.

In conclusion, our results suggest a high diagnostic usefulness of IgG ELISA based on a combination formula of SAG2, GRA2+GRA6+GRA7+GRA15 for detection of *T. gondii*

antibodies in cats sera. The current ELISA with M6 offers a promising tool for accurate serodiagnosis of toxoplasmosis in cats.

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Figure legend

Figure 1. Comparison between titers of sera by LAT with distribution of ELISA-ODs based on TLA (A) and recombinant combination formula M6 (B). Each circle indicates average OD of ELISA.

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Figure 1. (Abdelbaset et al., 2016)

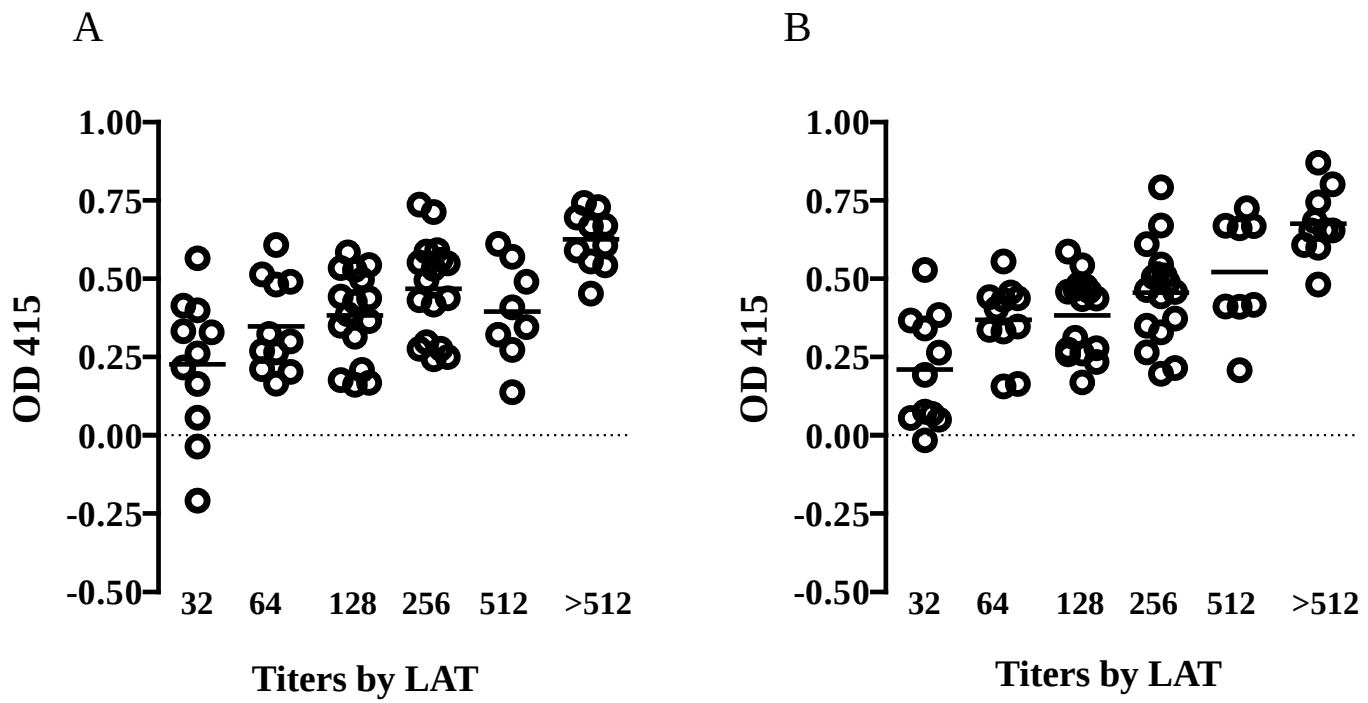

Table 1. Primers used for cloning of *T. gondii* genes

Genes	Primer sequence	Enzyme site
sag2	5'-ttttggatcctccaccaccgagacgcca-3'	*Bam*H1
	5'-ttttgaattcttacttgcccgtgagaga-3'	*Eco*R1
gra2	5'-ttggatccatgttcgccgtaaaacat-3'	*Bam*H1
	5'-ttctcgagttactgcgaaaagtctgg-3'	*Xho*1
gra6	5'-ttttggatccatggcacacggtggcatccatctga-3'	*Bam*H1
	5'-ttttgaattcataatcaaacacattcacacgttcc-3'	*Eco*R1
gra7	5'-ttttggatccgatgacgaactgatgagt-3'	*Bam*H1
	5'-ttttgaattcctactggcgggcatcctccccatctt-3'	*Eco*R1
gra15	5'-ttttggatccatggtgacaacaaccacgccaacgc-3'	*Bam*H1
	5'-ttttctcgagtggagttaccgctgattgtgtgtcc-3'	*Xho*1
mic10	5'-ttggatccagtctccaggcgtctattgg-3'	*Bam*H1
	5'-ttctcgagctacattgatttcctgcgtc-3'	*Xho*1

Table 2. Performance evaluation of IgG-ELISA with TLA and recombinant proteins as compared to LAT.

Antigen	Sensitivity (%)	Specificity (%)	Concordance (%)	Kappa value
SAG2	91.89	88.12	88.78	0.67
GRA2	27.03	96.52	84.25	0.30
GRA6	82.43	88.69	87.59	0.62
GRA7	35.1	89.86	80.19	0.27
GRA15	17.57	86.38	74.22	0.04
MIC10	16.21	85.8	73.51	0.02
TLA	97.29	93.62	94.27	0.93
M1	74.32	88.99	86.4	0.58
M2	44.59	89.28	81.38	0.35
M3	90.54	85.51	86.4	0.62
M4	94.59	89.57	90.45	0.72
M5	70.27	86.09	83.29	0.50
M6	89.19	95.36	94.27	0.81