FULL PAPER

Parasitology

Detection of antibodies against *Toxoplasma gondii* in cats using an immunochromatographic test based on GRA7 antigen

Rochelle Haidee D. YBAÑEZ[1], Hisako KYAN[2] and Yoshifumi NISHIKAWA[1]*

[1]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan
[2]Okinawa Prefectural Institute of Health and Environment, 17-1 Kanekadan, Uruma-shi, Okinawa 904-2241, Japan

ABSTRACT. Toxoplasmosis, caused by *Toxoplasma gondii*, is one of the most common parasitic diseases worldwide. The GRA7 of *T. gondii* (TgGRA7) is an essential component of the parasitophorous vacuole (PV) and PV membrane surrounding the tachyzoites and the cyst wall of the bradyzoites. While it has been widely employed as antigen for ELISA, there is only one study that has reported its potential as antigen for immunochromatographic test (ICT) in pigs. There is no study yet documenting its potential for ICT serodiagnosis of *T. gondii* infection in cats. In this study, we assessed the efficacy of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in 100 cats and compared the results with iELISAs using TgGRA7 and lysate antigens of *T. gondii* strains, RH, PLK, and VEG. Our results revealed that TgGRA7-ICT is a reliable test for the diagnosis of anti-*T. gondii* antibodies in cats, producing comparable results as conventional serological methods. This study is the first report on the use of TgGRA7 as ICT antigen for the serodiagnosis of *T. gondii* infection in cats.

KEY WORDS: cat, GRA7, immunochromatographic test, *Toxoplasma gondii*, toxoplasmosis

Toxoplasmosis is among the most common parasitic infections affecting humans and other warm-blooded animals and is caused by the intracellular apicomplexan parasite, *Toxoplasma gondii* [14]. *T. gondii* reproduces in the gut of cats, its known definitive hosts [8]. Oocysts are shed through the cat feces, which can be ingested by its intermediate hosts [23]. Postnatally, *T. gondii* infection in humans is acquired by ingestion of raw meat infected with tissue cysts, consumption of food and drink contaminated with oocysts, or by direct contact from the environment, such as the soil [9]. Vertical transmission of the parasite through the placenta from the infected mother to the fetus may also occur [19].

T. gondii infection in cats is generally asymptomatic and latent in nature [10]; however, it causes severe neurologic or ocular diseases in the fetus during pregnancy and in immunocompromised people [9]. Moreover, cat ownership and frequent contact with cats have been identified as significant risk factors for *T. gondii* infection in humans. Being the definitive host of *T. gondii*, cats have a primary role in the transmission of the parasite. And frequent contact with cats increases the risk of infection [2, 6, 25]. Therefore, the detection of *T. gondii* infection in cats is vital for diagnosis and control of toxoplasmosis. Frequently used diagnostic tests of *Toxoplasma* infection in humans and animals are based on the serological detection of specific antibodies such as the enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT) [13]. ELISA using *Toxoplasma* lysate antigens (TLAs) has been used as a diagnostic method of *T. gondii* infection; however, ELISA based on purified recombinant proteins is preferably used for routine diagnostic screenings and seroepidemiological surveys due to its easy test standardization and lesser production costs than TLAs [16, 20]. Moreover, the use of ICT based on recombinant antigens has gained popularity as it is fast, easy to use, economical, and can be used in the field [15, 22].

The dense granule antigen 7 of *T. gondii* (TgGRA7) is an essential component of the parasitophorous vacuole (PV) and PV membrane surrounding the tachyzoites and the cyst wall of the bradyzoites [3, 5, 18]. The effectiveness of TgGRA7 as a serodiagnostic marker for *T. gondii* infection have already been confirmed using indirect ELISA (iELISA), with a sensitivity of 81 to 98.9% and specificity of 98 to 100% [11, 16, 17] in humans, and 94.9% sensitivity and 97.9% specificity in cats [4]. Whereas TgGRA7 has been widely employed as antigen for ELISA, there is only one study that has documented its potential as antigen

for ICT. Test results of the serological detection of *T. gondii* infection in pigs using ICT based on TgGRA7 were highly sensitive and specific and were substantially concordant with the results of latex agglutination test (LAT) and TgGRA7-based iELISA [22]. There is no study yet reporting its potential as an antigen for ICT serodiagnosis of *T. gondii* infection in cats.

In this study, we evaluated the efficacy of an ICT using TgGRA7 in the detection of *Toxoplasma* infections in cats and compared the results with iELISAs using TgGRA7 and lysate antigens of *T. gondii* strains, RH, PLK, and VEG. Our results revealed that TgGRA7-ICT is a reliable test for the diagnosis of anti-*T. gondii* antibody in cats, producing comparable results as conventional serological methods.

MATERIALS AND METHODS

Ethical clearance

This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocol was approved by the Committee on the Ethics of Animal Experiments at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan (permit number 19-3).

Cat serum samples

In this study, a total of 100 cat serum samples were collected from Animal Welfare and Management Center, Okinawa, Japan. The sera were stored at −30°C until further use.

Recombinant TgGRA7 preparation

Total RNA from the pelleted RH strain of *T. gondii* was isolated, and cDNA was synthesized and amplified as previously described [22]. The recombinant protein of TgGRA7 (rTGRA7) was expressed as a glutathione S-transferase (GST) fusion protein in the *E. coli* DH5α strain (Takara Bio, Inc., Kusatsu, Japan). The GST tag of the rTGRA7 was cut using thrombin protease (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The rTGRA7 is a 29 kDa protein as confirmed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

T. gondii culture and lysate antigen preparation

T. gondii tachyzoites from the RH, PLK, and VEG strains were maintained in African green monkey kidney (Vero) cells cultured in Eagle's Minimum Essential Medium (EMEM, Sigma, St. Louis, MO, USA) supplemented with 8% heat-inactivated fetal bovine serum (FBS). For purification of tachyzoites, the infected cells were washed with cold phosphate-buffered saline (PBS). Cell pellets were resuspended in medium and passed through a 27-gauge needle and then through a 5.0-μm-pore filter (Millipore, Bedford, MA, USA). After centrifugation at 10,000 ×g for 10 min at 4°C, the pellet was resuspended in cold PBS and sonicated at 3 × 30 sec at 5 kHz (Iwaki Ultrasonic Cleaner, Tokyo, Japan). The cells were freeze-thawed three times and centrifuged again. The supernatant was collected, and the concentration of the lysates was measured using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The TLAs were kept at −30°C until use.

iELISA using TgGRA7 and TLAs

The 96-well ELISA plates were coated overnight at 4°C with 50 μl of the recombinant TgGRA7 and the TLAs diluted to a final concentration of 0.1 μM and 2 μg/m*l*, respectively, using 50 mM carbonate-bicarbonate buffer (pH 9.6) as previously described [1, 21]. The cat sera were diluted at 1:500, and the secondary antibody used was horseradish peroxidase-conjugated anti-cat IgG (Sigma-Aldrich, St. Louis, MO, USA) diluted at 1:4,000. The serum samples and secondary antibody were incubated for 1 hr at 37°C, while the subtrate was incubated at room temperature for 1 hr. The cutoff points were determined as the mean value at an optical density of 415 nm (OD_{415}) plus three standard deviations (0.1738 for anti-TgGRA7, 0.1801 for anti-TgRH, 0.1462 for anti-TgPLK, and 0.1641 for anti-TgVEG) of cat sera (N=24) previously tested negative using a commercial LAT kit (Toxocheck-MT; Eiken Chemical, Tokyo, Japan).

TgGRA7-ICT preparation

The ICT strips were prepared as previously described [12, 22]). The recombinant TgGRA7 and purified rabbit anti-TgGRA7 IgG antibody [22] used were at a concentration of 1 mg/m*l*. The assay was performed by pipetting 30 μl of undiluted cat serum samples to the sample pad. The results were judged within 20 min of band appearance at the control and/or test line (Fig. 1).
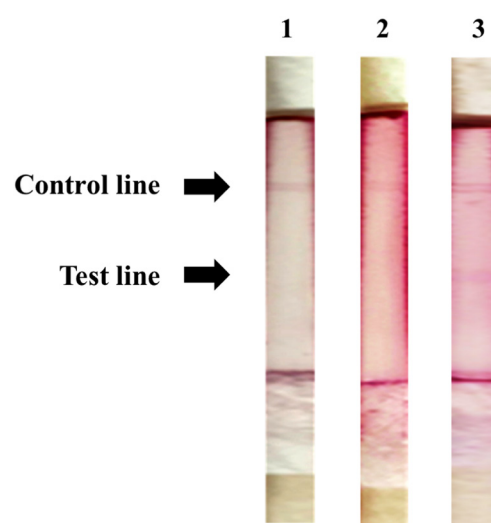

Fig. 1. Representative images of the immunochromatographic test using TgGRA7 reactions. Strip 1: phosphate-buffered saline, strip 2: *Toxoplasma gondii*-negative cat serum, strip 3: *T. gondii*-positive cat serum.

Statistical analysis

The sensitivity, specificity, and kappa values with a 95% confidence interval were calculated using an online statistical tool (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 very good (0.81 to 1.00). The correlation coefficients between relative intensity in the ICT band and absorbance values in the ELISA were calculated using Pearson's correlation coefficient. To calculate the relative ICT band intensity, ICT pictures were converted into 8-bit JPEG images, then the intensity of the grayscale images was analyzed using ImageJ software v. 1.49 (Mac version of NIH Image, http//rsb.info.-nih.gov/nih-image/) as described [12].

RESULTS

In this study, we confirmed the effectiveness of the *Toxoplasma* ICT using TgGRA7 for serodiagnosis in cats by testing 100 cat serum samples. The results were compared with the results obtained using a commercial LAT kit, iELISA based on TgGRA7, and iELISAs using *T. gondii* RH, PLK, and VEG lysate antigens. Table 1 shows that our ICT using TgGRA7 detected a total of 74 positive samples, which is similar to the result obtained by iELISA using TgGRA7. There were two samples that tested positive by LAT but were negative for ICT. Moreover, 71 cat sera showed positive by iELISA using RH lysate while 70 samples were positive using PLK and VEG lysates. Generally, our TgGRA7-ICT had a sensitivity of 97.4–100% and specificity of 89.7–100% for the detection of IgG antibodies (Table 2). Using TgGRA7-iELISA as a reference test, we obtained 100% sensitivity and specificity for our TgGRA7-ICT. When LAT was used as reference, our ICT showed a sensitivity of 97.4% and specificity of 100%. Meanwhile, using the lysate-based iELISAs, we obtained 100% and 87.7–89% sensitivity and specificity, respectively. With kappa values ranging from 0.901 to 1 (Table 2), the results of our TgGRA7-ICT were in very good agreement with the results of the different iELISAs and LAT. Furthermore, to confirm the reliability of test results using TgGRA7-ICT, the correlation between the ICT band intensity and ELISA OD values was analyzed (Fig. 2). The analysis revealed a strong correlation between the relative band intensity in the TgGRA7-ICT and absorbance values in the GRA7-iELISA (Pearson's r=0.8957). Overall, the ICT results obtained in this study are similar to the results using conventional serological methods. This suggests that an ICT based on TgGRA7 can be used for serodiagnosis of *T. gondii* infection in cats.

DISCUSSION

Cats are important in the transmission of *T. gondii* through the shedding of the infectious oocysts in their feces which may contaminate the environment. Serological testing such as the ICT confirms infection in cats; thus, they can be brought to animal hospitals where appropriate medication can be started. As for seronegative cats, precautionary measures could be done to reduce the risk of infection including keeping the cats indoors to avoid hunting of possibly-infected rodents and other animals, avoiding raw or undercooked meat in the cat's diet, and disposing and cleaning cat litters regularly to minimize chances of exposure to infective oocysts and shedding the oocysts into the environment.

The TgGRA7-ICT developed in this study demonstrated an excellent diagnostic capability in the detection of anti-*Toxoplasma* antibodies in cat serum samples, showing high specificity and sensitivity, and very good agreement with the results of current

Table 1. Results of cat sera tested by TgGRA7-immunochromatographic test (ICT), TgGRA7-indirect enzyme-linked immunosorbent assay (iELISA), *Toxoplasma* lysate antigen-iELISAs, and latex agglutination test (LAT) (N=100)

TgGRA7-ICT	iELISA								LAT		Total
	TgGRA7		RH		PLK		VEG				
	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	
Negative	26	0	26	0	26	0	26	0	24	2	26
Positive	0	74	3	71	4	70	4	70	0	74	74
Total	26	74	29	71	30	70	30	70	24	76	100

Table 2. Sensitivity and specificity of the TgGRA7-immunochromatographic test (ICT) in the detection of specific *Toxoplasma gondii* antibodies in cat sera compared with the results of the TgGRA7-indirect enzyme-linked immunosorbent assay (iELISA), *Toxoplasma* lysate antigen-iELISAs, and latex agglutination test (LAT)

Parameter	iELISA				LAT
	GRA7	RH	PLK	VEG	
Sensitivity (%)	100	100	100	100	97.4
Specificity (%)	100	89.7	87.7	87.7	100
Kappa value	1	0.943	0.901	0.901	0.947

The parameters were calculated using an online statistical tool (www.vassarstats.net). The strength of agreement (kappa value) between each test and the ICT was graded as fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and very good (>0.90).
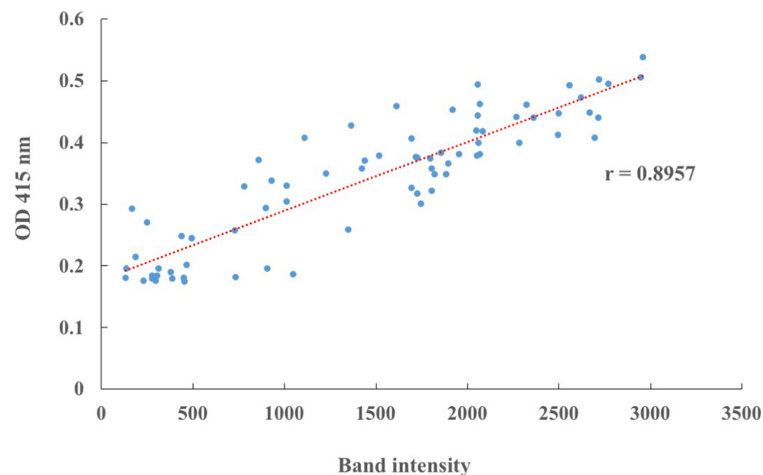
Fig. 2. Correlation between immunochromatographic test (ICT) band intensity and enzymed-linked immunosorbent assay (ELISA) OD values. Scatter plot shows the correlation between relative intensity in the ICT and absorbance values in the ELISA using cat serum samples. The break line represents the calculated line of best fit. Correlation coefficients were calculated using Pearson's correlation coefficient: |r|=0.70, strong correlation; 0.5<|r|<0.7, moderately strong correlation; and |r|=0.3–0.5 weak to moderate correlation.

serodiagnostic methods. These results were supported by previous studies showing the usefulness of TgGRA7 as a serological marker for the detection of IgG in acute and chronic infections in humans [16, 18] and animals, including dogs [24] and cats [4]. The strong antigenic characteristics of TgGRA7 could be due to its abundance on the surface of host cells and within the PV, the PVM, and the host cell cytosol and its expression in all infectious stages of *T. gondii* [3, 5, 18]. Direct contact of TgGRA7 with the host immune system following its release by the tachyzoites and bradyzoites after host cell rupture prompts a strong antibody response in both early and late stages of infection [7, 16, 21].

In the present study, we were able to develop a highly reliable ICT based on TgGRA7 for the detection of antibodies against *T. gondii* infection in cats. The GRA7-ICT is fast, easy to use, affordable, and can be easily standardized in comparison with the current serological tests. Thus, this can be used as an alternative test for routine screening of *T. gondii* in cats. This study is the first report on the use of TgGRA7 as an ICT antigen for the serodiagnosis of *T. gondii* infection in cats.

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