ISSN (Print) 0023-4001
ISSN (Online) 1738-0006

Korean J Parasitol Vol. 49, No. 3: 207-212, September 2011
http://dx.doi.org/10.3347/kjp.2011.49.3.207

Development and Clinical Evaluation of a Rapid Serodiagnostic Test for Toxoplasmosis of Cats Using Recombinant SAG1 Antigen

Chom-Kyu Chong[1,†], Wooseog Jeong[2,†], Hak-Yong Kim[1], Dong-Jun An[2], Hye-Young Jeoung[2], Jeong-Eun Ryu[2], A-Ra Ko[3], Yong-Joo Kim[3], Sung-Jong Hong[4], Zhaoshou Yang[5] and Ho-Woo Nam[5,*]

[1]Department of Biochemistry, College of Natural Science, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea; [2]Bacteriology and Parasitology Division, National Veterinary Research and Quarantine Service, Anyang 430-824, Korea; [3]Institute of BioMedicals, Bioland Ltd., Ochang, Chungbuk 363-883, Korea; [4]Department of Medical Environmental Biology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea; [5]Department of Parasitology and Catholic Institute of Parasitic Diseases, College of Medicine, Catholic University of Korea, Seoul 137-701, Korea

Abstract: Rapid serodiagnostic methods for *Toxoplasma gondii* infection in cats are urgently needed for effective control of transmission routes toward human infections. In this work, 4 recombinant *T. gondii* antigens (SAG1, SAG2, GRA3, and GRA6) were produced and tested for the development of rapid diagnostic test (RDT). The proteins were expressed in *Escherichia coli*, affinity-purified, and applied onto the nitrocellulose membrane of the test strip. The recombinant SAG1 (rSAG1) showed the strongest antigenic activity and highest specificity among them. We also performed clinical evaluation of the rSAG1-loaded RDT in 182 cat sera (55 household and 127 stray cats). The kit showed 0.88 of kappa value comparing with a commercialized ELISA kit, which indicated a significant correlation between rSAG1-loaded RDT and the ELISA kit. The overall sensitivity and specificity of the RDT were 100% (23/23) and 99.4% (158/159), respectively. The rSAG1-loaded RDT is rapid, easy to use, and highly accurate. Thus, it would be a suitable diagnostic tool for rapid detection of antibodies in *T. gondii*-infected cats under field conditions.

Key words: *Toxoplasma gondii*, cat, rapid diagnostic test, serodiagnosis, recombinant SAG1

INTRODUCTION

Toxoplasma gondii is an apicomplexan parasite with a broad host range of most warm-blooded animals from mammals to birds [1]. The protozoan parasite can cause congenital defects or abortion and neonatal complications in immune compromised patients [2]. Cats are the final host of this pathogen because they are the unique species that excretes infectious oocysts [3,4]. Oocysts can be introduced into humans while gardening or consuming fresh products or drinking water contaminated with the oocysts. Because *T. gondii* persists in pigs, goats, and other mammals as the tissue-cyst forms after infection by the oocyst, humans can be infected by consuming insufficiently cooked meats carrying the tissue cysts also. Another route of infection is from infected mother to her fetus via placental transmission of *T. gondii*, a process known as congenital toxoplasmosis. The congenital toxoplasmosis may cause stillbirth or abortion in addition to serious damages to the fetus, such as severe neurological disorders after delivery [5]. For these reasons, control of *T. gondii* transmission from cats is one of the best ways to prevent its infection to humans. Monitoring of *T. gondii*-specific antibodies in cats is important because the detection of oocysts or direct antigens is virtually impossible in the field conditions.

Several serological methods have been developed for detection of *T. gondii* infection [6-8]. ELISA and latex agglutination test (LAT) are popularly and commercially available for the serodiagnosis of feline toxoplasmosis and western blot analysis is normally used for confirmation. However, the procedures of these methods are laborious and time-consuming and require expertise and equipments, rendering them from point-of-care testing. To overcome these disadvantages, detection method in the form of rapid diagnostic test (RDT) have been introduced. Recently, a truncated SAG2-loaded RDT was developed and

• Received 25 April 2010, revised 23 June 2011, accepted 27 June 2011.
* Corresponding author (howoo@catholic.ac.kr)
† Chom-Kyu Chong and Wooseog Jeong equally contributed as the first author.

evaluated for its diagnostic properties on infected and uninfected cats [9]. Nevertheless, the potential of other antigens of *T. gondii* to be used in diagnosis as an RDT system is largely unknown. To our knowledge, the crude extract or somatic antigens of *T. gondii* was generally applied as raw materials of diagnostic systems, including ELISA and LAT [10,11]. However, the use of these biomaterials can evoke several diagnostic problems, such as false positives, cross-reactions, or lot-to-lot variations of the kits [12]. To solve these matters, recombinant proteins which are specific to *T. gondii* antibodies have been introduced. Several antigens of *T. gondii* have been studied and reported [13-15]. Among them, surface antigens (SAG) and dense granule proteins (GRA) are mostly used as antigenic materials of the diagnostic kit to detect antibodies against *T. gondii*.

In this study, we investigated the antigenic properties as well as the suitability of 4 recombinant proteins, SAG1, SAG2, GRA3, and GRA6, to be used in the development of RDT for *T. gondii*. We then developed a recombinant SAG1 (rSAG1)-based RDT kit which could specifically detect *T. gondii* antibodies in cat blood. Finally, we evaluated its serodiagnostic performances using clinical specimens from household and stray cats in Korea.

MATERIALS AND METHODS

Clinical samples

A total of 182 cases of feline sera (55 cases of household and 127 cases of stray cats) were kindly provided by the National Veterinary Research and Quarantine Service (NVRQS, Anyang, Korea). The sera were collected in Gyeonggi-do and Jeollabuk-do provinces in Korea from 2009 to 2010. All sera were examined by RDT kit and the results were compared with those of a commercially available ELISA kit (ID Screen® Toxoplasmosis Indirect, ID VET Inc., Montpellier, France).

Preparation of recombinant proteins from *T. gondii*

Recombinant proteins of SAG1, SAG2, GRA3, and GRA6 were produced in BL21 (DE3) strain of *E. coli* to test the antigenicity against *T. gondii* antibodies. Briefly, cells containing the expression vector for each protein were grown at 37℃ in LB medium to an OD_{600} of 0.7-0.8. Protein expression was induced with 0.5 mM IPTG for 4 hr at 30℃. The pellet was resuspended in the standard buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM EDTA and 1 mM PMSF) and sonicated on ice. The supernatant after centrifugation at 20,000 *g* for 20 min was applied on a GSH-coupled Sepharose 6B column. The bound GST-fused recombinant protein was recovered with the elution buffer (50 mM Tris-HCl, pH 7.5, 15 mM GSH). The purified protein was identified by SDS-PAGE analysis, and protein concentration was determined by the method of Bradford [16].

Preparation of colloidal gold and conjugation with recombinant proteins

Colloidal gold particles (40 nm mean diameter) were prepared according to the procedure described [17]. Briefly, 100 ml of 0.01% $HAuCl_4$ solution was boiled thoroughly. Then, 1.8 ml of 1% trisodium citrate solution was added under constant stirring. After the color change to wine-red for about 45 sec, it was allowed to boil again for another 5 min. Then, the colloidal gold solution was stirred for additional 10 min at room temperature (RT) and stored in a dark bottle at 4℃ until used. The recombinant antigens of *T. gondii* (rTgAg) were conjugated with 40-nm colloidal gold particles as described previously [18]. Briefly, rTgAg dialyzed against PBS and the gold solution adjusted to pH 9.0 with 0.2M K_2CO_3 were mixed at the ratio of 1 mg protein to 100 ml gold solution (≈2.0 at OD_{540}). The mixture was incubated for 30 min at RT and then blocked with 1% BSA for 30 min. The conjugate was then washed with PBS containing 1% BSA and the OD of rTgAg-gold conjugate was measured at 450 nm.

Preparation of strip for rapid diagnostic test (RDT)

The composition of RDT device to detect *T. gondii*-specific antibodies (named as "TgRDT" in this report) is shown in Fig. 1. The test device was prepared as follows: recombinant protein as capturer was immobilized to an appropriate position

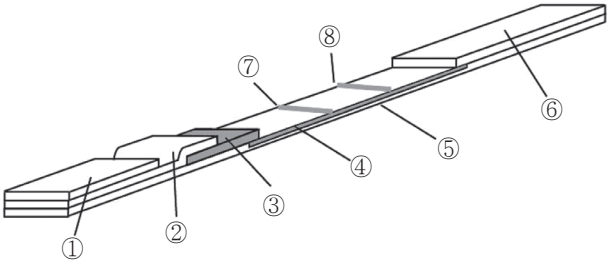

Fig. 1. Diagram of TgRDT strip to detect antibodies against *T. gondii*. rTgAg was immobilized onto the T region and anti-mouse IgG was onto the C area of the strip. Conjugate pad contains rTgAg- and mouse IgG-conjugated gold colloid. ① sample pad; ② blood separation pad; ③ conjugate pad; ④ nitrocellulose membrane; ⑤ plastic backbone; ⑥ absorbance pad; ⑦ test line (T); and ⑧ control line (C).

of the nitrocellulose membrane (control line [C]; 1.0 mg/ml of goat anti-mouse IgG, test line [T]; 1.0 mg/ml of rTgAg), and then the membrane was dried at RT. The rTgAg- and mouse IgG-conjugated gold colloid was treated simultaneously, dried on a glass fiber, and used as the conjugate pad. Sample pad was prepared from the treatment of cellulose paper with 0.1M Tris-Cl (pH 8.0) containing 0.5% polyvinylalcohol (PVA) and 0.5% Tween-20. For the absorbance pad, the cellulose paper was used without any treatment. The test strip was assembled in the order shown in Fig. 1. All pads were partially overlapped to ensure uninterrupted migration of the sample solution along the test strip. The test strip was finally assembled in a plastic cassette.

Assay procedure and interpretation of TgRDT

As shown in Fig. 2A, when the test strip is assembled in a plastic cassette, there is one hole which connects to the sample pad. The assay procedure started by loading 10 µl of blood sample onto the hole, and then loading 3 drops (approximately 100 µl) of 0.1M Tris-Cl buffer (pH 8.0) containing 0.1% casein and 1% Tween-20 to the sample pad. Approximately 5-10 min after the application of the buffer, the results were interpreted. Control line (C) should appear in all tests. If a red color band appears at the T line, it means that the specimen contains anti-*T. gondii* antibodies (Fig. 2A).

Analytical sensitivity of TgRDT

To investigate the analytical sensitivity of TgRDT, we used 3 different positive specimens. The sera from cats were serially diluted with negatives ranging from 1 to 128 folds and then they were used to the testing. The detection threshold level of the kit was compared with the commercial ELISA kit, ID Screen® Toxoplasmosis Indirect, which has been used popularly in Korea. The S/*P* values were calculated by the following formula according to the direction of the kit insert.

$$S/P = \frac{\text{Optical density of sample } (OD_{sample})}{\text{Optical density of positive control } (OD_{pc})} \times 100$$

If the S/*P* value is greater than or equal to 50%, the sample is considered positive to anti-*T. gondii* antibodies. Otherwise, the sample is considered negative or doubtful.

Clinical assessment of TgRDT

Total 182 cases of feline specimens were used for evaluating

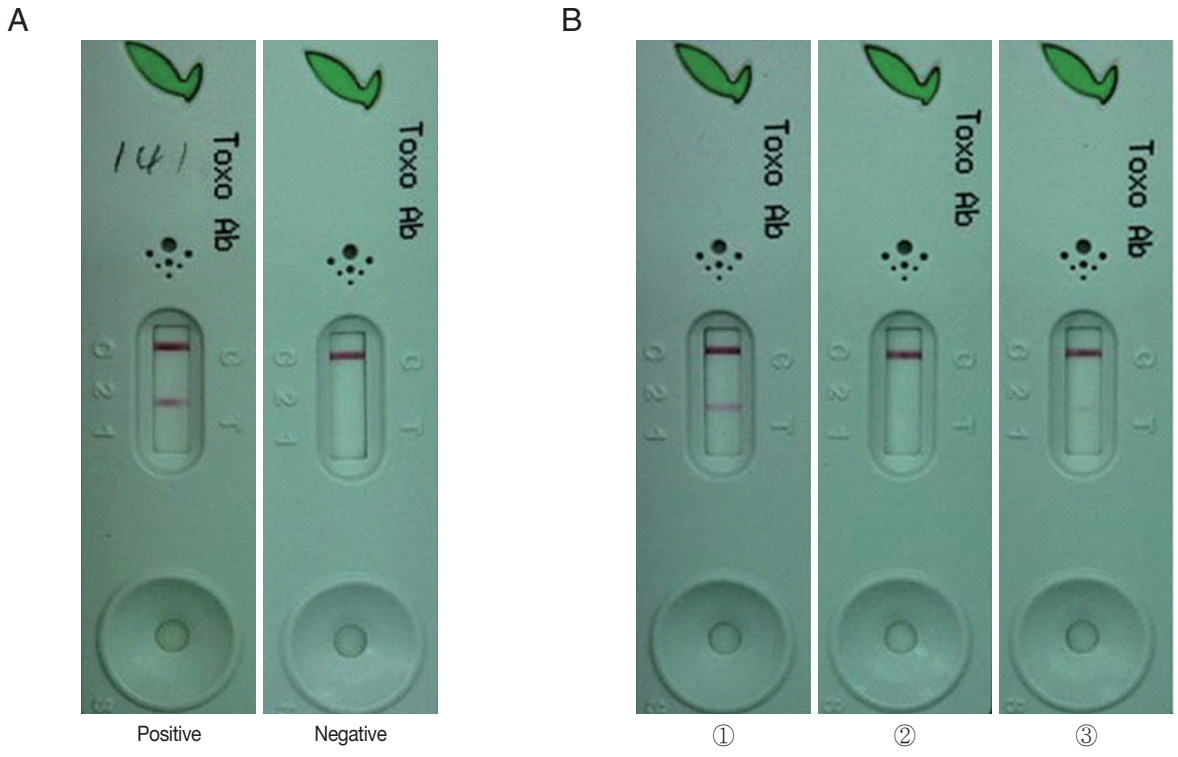

Fig. 2. (A) Positive and negative results of the assembled TgRDT kit. (B) Immuno-reactivities of rSAG1, rSAG2, and rGRA6 to antibodies against *T. gondii*. ① rSAG1-applied; ② rSAG2-applied; and ③ rGRA6-applied.

the TgRDT developed in this study. All the samples were comparatively tested by the commercial ELISA kit. For confirmation of the samples, western blot analysis was carried out. Clinical accuracy of the TgRDT kit, including sensitivity, specificity, and kappa values was calculated by the general diagnostic formulas [19].

RESULTS

Antigenic properties of the recombinant proteins from *T. gondii*

Among the 4 kinds of rTgAg, namely SAG1, SAG2, GRA3, and GRA6, GRA3 was expressed as the inclusion body, hence, the protein was excluded from further analysis (data not shown). The remaining 3 recombinant proteins were successfully expressed and purified. Then, the antigens were applied to RDT system as described previously. As shown in Fig. 2B, SAG2 and GRA6 showed weak reactivity to the anti-*T. gondii* antibody-positive serum from cats. To check the overall antigenic reactivities of the proteins with respect to various positive sera, we tested with mixture of 10 kinds of positive sera. Among the antigens tried, SAG1 gave very strong antigenic reactivity to the positive serum so that we introduced this protein to the TgRDT kit in this study.

Analytical sensitivity of TgRDT with SAG1 protein

To compare the detection threshold levels of TgRDT and ELISA kit, 3 different sera had been verified to be positive for anti-*T. gondii* antibody by western blot (data not shown). The positive sera were serially diluted with the verified negative serum. As shown in Table 1, the detection threshold levels using those diluents were 38.2, 24.7, and 68.9 S/P for TgRDT, and 93.1, 97.9, and 68.9 for ELISA, respectively. These data indicate that the cut-off value of TgRDT (43.9 S/P) is approximately ~2-folds lower than that of ELISA (86.6 S/P).

Table 1. Analytical sensitivity of TgRDT and ELISA

Positive sample (ID)	Dilution fold	S/*P* value	Detection thresholds	
			TgRDT	ELISA
No. 1 (#33)	4	132	+	+
	16	125	+	+
	64	69	+w	+
	128	42	+w	-
	256	24	-	-
No. 2 (#141)	4	140	++	+
	16	98	+	+
	64	49	+	-
	128	25	+w	-
	256	16	-	-
No. 3 (#154)	4	134	+	+
	16	93	+	+
	64	38	+w	-
	128	23	-	-

There were no discrepancies between observations made by 2 technicians.
+, positive; +w, very weak positive; and -, negative.

Clinical accuracy of rSAG1-loaded RDT

Total 55 household and 127 stray feline sera were tested in this clinical study. As shown in Table 2, the TgRDT has showed excellent and promising clinical performances. Its sensitivity was 100% (23/23) even though the number of positive samples was limited, and its specificity was 99.2% (158/159). In the case of ELISA kit, it showed 91.3% of clinical sensitivity (21/

Table 2. Clinical performance of TgRDT and ELISA

Reference results	TgRDT			ELISA		
	Positive	Negative	Total	Positive	Negative	Total
Positive	23	0	23	21	2	23
Negative	1	158	159	2	157	159
Total	24	158	182	23	159	182

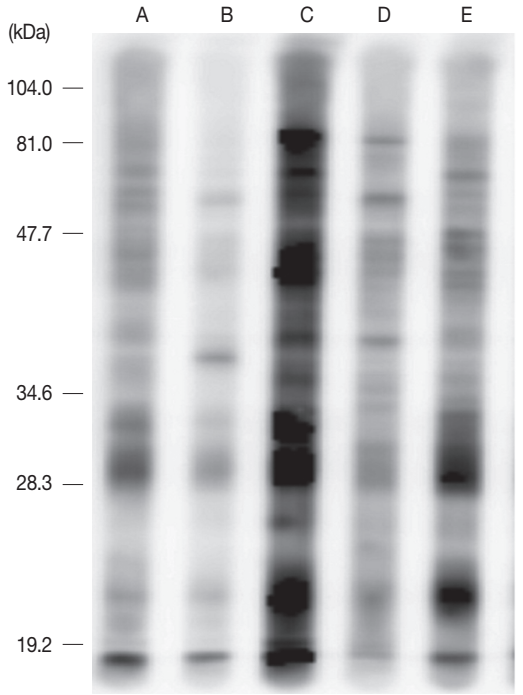

Fig. 3. Western blot analysis of 5 specimens having different results between TgRDT and ELISA.

23) and 98.7% of clinical specificity (157/159). The kappa value of these 2 methods was 0.88. Five cases of cat sera showed different results between TgRDT and ELISA. Fig. 3 is the results of western blot analysis for confirmation, of which 2 cases (C and E) showed typical band patterns of *T. gondii* positive serum and the other 3 cases were negative. All the results were analyzed following the method of Sohn and Nam [20].

DISCUSSION

There are several recombinant proteins of *T. gondii* known as potential candidates for the diagnostic use to detect its antibodies in cats and also in humans. Among them, SAG1 is one of the most suitable proteins for diagnostic system because of its structural abundance in *T. gondii* and better antigenic properties. In this study, we demonstrated that rSAG1 effectively detected *T. gondii* antibodies in cats, and that the recombinant protein could replace somatic antigens of *T. gondii* in the diagnosis. The rSAG1-loaded TgRDT showed very good analytical sensitivity in our experiments (2-fold more sensitive than ELISA kit). Furthermore, the high analytical sensitivity and the use of recombinant protein produced excellent results of clinical assessment of TgRDT (100% of sensitivity and 99.2% of specificity).

According to the algorithm of kappa statistics, the kappa value shall range from 0 to 1, and a kappa value of more than 0.7 typically indicates strong agreement of 2 methods [19]. In our clinical trial, Cohen's kappa value of TgRDT and a commercial ELISA kit was 0.88 indicating that both kits have very good concordance to detect the antibodies against *T. gondii*. This result also suggests that the recombinant SAG1 can be used as a choice of diagnostic antigen instead of somatic antigens, such as *T. gondii* lysate without compromise.

In the field trial of this study, cat sera collected in Gyeonggido and Jeollabuk-do provinces showed 12.6% of Tg Ab-positive rates (23/182), but there was a significant difference in the Tg Ab-positive rates between household and stray cats. Household cats showed 1.8% of Tg Ab positive rate (1/55) but stray cats gave 17.3% of Tg Ab positive rate (22/159), which showed compatible positive rates done earlier [21-23] and suggested overall prevalence of *T. gondii* infection in Korean cats. This reflects the pet-loving behavior of Korean people as clean as possible and severely restricting the physical activity of cats within house. Therefore, the household cat is well controlled from *T. gondii* infection whereas the stray cats are exposed to more harsh environment to be infected with *T. gondii* and may function as transmitter of this parasite to the other cats and intermediate animals according to the due infection routes.

In conclusion, to our knowledge, this TgRDT is the first rSAG1-based RDT kit to diagnose *T. gondii*-specific antibodies. The kit is simple to use, rapid to assay, and very sensitive and highly specific. Therefore, it would serve as a choice of method for point-of-care diagnosis of *T. gondii* infection in cat under clinical or field conditions. Further studies are ongoing to device for the diagnostic utility of *T. gondii* infection in humans and for discrimination of IgM and IgG against *T. gondii*.

ACKNOWLEDGEMENTS

This project was supported by a grant from the National Veterinary Research and Quarantine Service in Korea, 2010.

REFERENCES

1. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: From animal to humans. Int J Pathol 2000; 30: 1217-1258.
2. Remington JS, McLeod R, Thulliez P, Desmonts G. Toxoplasmosis. In Remington JS, Klein J eds, Infectious Diseases of the Fetus and Newborn Infant, 5th ed. Philadelphia, USA. WB Saunders. 2001, p 205-346.
3. Omata Y, Oikawa H, Kanda M, Mikazuki K, Nakabayashi T, Suzuki N. Experimental feline toxoplasmosis: Humoral immune responses of cats inoculated orally with *Toxoplasma gondii* cysts and oocysts. J Vet Med Sci 1990; 52: 865-867.
4. Frenkel JK. Biology of *Toxoplasma gondii*. In Ambroise-Thomas P, Peterse E eds, Congenital Toxoplasmosis: Scientific Background, Clinical Management and Control. Paris, France. Springer-Verlag. 2000, p 9-25.
5. Wong SY, Remington JS. Toxoplasmosis in pregnancy. Clin Infect Dis 1994; 18: 853-861.
6. Liesenfeld O, Press C, Flanders R, Ramirez R, Remington JS. Study of Abbott Toxo IMx system for detection of immunoglobulin G and immunoglobulin M *Toxoplasma* antibodies: Value of confirmatory testing for diagnosis of acute toxoplasmosis. J Clin Microbiol 1996; 34: 2526-2530.
7. Jenum PA, Stray-Pedersen B. Development of specific immunoglobulins G, M, and A following primary *Toxoplasma gondii* infection in pregnant women. J Clin Microbiol 1998; 36: 2907-2913.
8. Liesenfeld O, Press C, Montoya JG, Gill R, Isaac-Renton JL, Hedman K, Remington JS. False-positive results in immunoglobulin M (IgM) toxoplasma antibody tests and importance of confirmatory testing: The Platelia Toxo IgM test. J Clin Microbiol 1997; 35: 174-178.
9. Huang X, Xuan X, Hirata H, Yokoyama N, Xu L, Suzuki N, Iga-

rashi I. Rapid immunochromatographic test using recombinant SAG2 for detection of antibodies against *Toxoplasma gondii* in cats. J Clin Microbiol 2004; 42: 351-353.
10. Choi WY, Yoo JE, Nam HW, Oh CY, Kim SW, Katakura K, Kobayashi A. *Toxoplasma* antibodies by indirect latex agglutination tests in zoo animals. Korean J Parasitol 1987; 25: 13-23.
11. Choi WY, Nam HW, Youn JH, Kim DJ, Kong Y, Kang SY, Cho SY. Detection of antibodies in serum and cerebrospinal fluid to *Toxoplasma gondii* by indirect latex agglutination test and enzyme-linked immunosorbent assay. Korean J Parasitol 1992; 30: 83-90.
12. Hassl A, Müller WA, Aspöck H. An identical epitope in *Pneumocystis carinii* and *Toxoplasma gondii* causing serological cross reactions. Parasitol Res 1991; 77: 351-352.
13. Pfrepper KI, Enders G, Gohl M, Krczal D, Hlobil H, Wassenberg D, Soutschek E. Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. Clin Diagn Lab Immunol 2005; 12: 977-982.
14. Carvalho FR, Silva DA, Cunha-Júnior JP, Souza MA, Oliverira TC, Béla SR, Faria GG, Lopes CS, Mineo JR. Reverse enzyme-linked immunosorbent assay using monoclonal antibodies against SAG1-related sequence, SAG2A, and p97 antigens from *Toxoplasma gondii* to detect specific immunoglobulin G (IgG), IgM, and IgA antibodies in human sera. Clin Vaccine Immunol 2008; 15: 1265-1271.
15. Buffolano W, Beghetto E, Del Pezzo M, Spadoni A, Di Cristina M, Petersen E, Gargano N. Use of recombinant antigens for early postnatal diagnosis of congenital toxoplasmosis. J Clin Microbiol 2005; 43: 5916-5924.
16. Kruger NJ. The Bradford method for protein quantitation. Methods Mol Biol 1994; 32: 9-15.
17. Frens G. Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature (London) 1973; 241: 20-22.
18. van den Brink W, van der Loos C, Volkers H, Lauwen R, van den Berg F, Houthoff HJ, Das PK. Combined beta-galactosidase and immunogold/silver staining for immunohistochemistry and DNA in situ hybridization. J Histochem Cytochem 1990; 38: 325-329.
19. Jacobson RH. Validation of serological assays for diagnosis of infectious diseases. Manual of Standards for Diagnostic Tests and Vaccines. France. OIE. 1996, p 8-15.
20. Sohn WM, Nam HW. Western blot analysis of stray cat sera against *Toxoplasma gondii* and the diagnostic availability of monoclonal antibodies in sandwich-ELISA. Korean J Parasitol 1999; 37: 249-256.
21. Kim HY, Kim YA, Kang S, Lee HS, Rhie HG, Ahn HJ, Nam HW, Lee SE. Prevalence of *Toxoplasma gondii* in stray cats of Gyeonggido, Korea. Korean J Parasitol 2008; 46: 199-201.
22. Lee SE, Kim JY, Kim YA, Cho SH, Ahn HJ, Woo HM, Lee WJ, Nam HW. Prevalence of *Toxoplasma gondii* infection in stray and household cats in regions of Seoul, Korea. Korean J Parasitol 2010; 48: 267-270.
23. Lee SE, Kim NH, Chae HS, Cho SH, Nam HW, Lee WJ, Kim SH, Lee JH. Prevalence of *Toxoplasma gondii* infection in feral cats in Seoul, Korea. J Parasitol 2011; 97: 153-155.