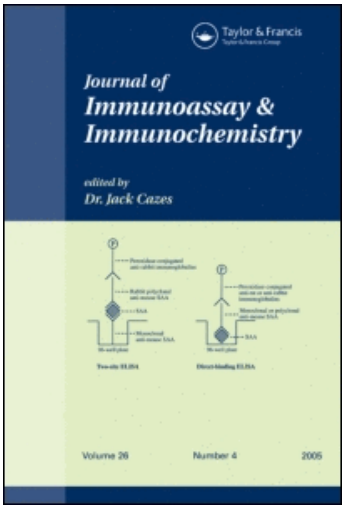

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AN ASSAY FOR SELECTION OF SERA WITH CIRCULATING *TOXOPLASMA GONDII* ANTIGENS

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□ *We have developed an ELISA that employs monoclonal anti-*Toxoplasma *SAG1 (p30) as the capture antibody to detect* T. gondii *circulating antigens in patients' serum samples. Using serum spiked with* Toxoplasma *soluble and with SAG1 recombinant proteins, the detection limits were 31.25 ng/mL and 62.50 ng/mL, respectively. We obtained positive results in 28% (21/75) and 11% (23/206) of probable active and chronic toxoplasmosis serum samples, respectively. Western blot analysis on pooled antigen-positive serum samples showed antigenic bands of molecular weights 25 and 75 kDa from sera of probable active infection and five antigenic bands ranging in size from 26 to 33 kDa from chronic infection sera. This assay would be useful as an initial serum selection step in developing a* Toxoplasma *antigen detection test and for characterization studies.*

Keywords circulating antigen, ELISA, *Toxoplasma gondii*, Western blot

INTRODUCTION

Toxoplasmosis is a ubiquitous infection caused by the protozoan parasite, *Toxoplasma gondii*. This infection produces little to no clinical symptoms in immunocompetent individuals. However, severe *T. gondii* infections can occur as primary infections during pregnancy, where it carries the risk of congenital

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toxoplasmosis; and in patients with cellular immunodeficiency, such as AIDS and in transplant patients. In Malaysia, the overall seroprevalence of toxoplasmosis among pregnant women was reported to be 49% of which 39%, 4%, and 6% were positive for anti-*Toxoplasma* IgG, IgM and both IgG and IgM antibodies, respectively.[1] In most cases, it is the acute/active infection that is important to enable the clinician to decide on commencement of treatment. Chronic infection does not require treatment; however, it may convert to an active infection in immunocompromised patients. Therefore, the availability of a panel of early diagnostic tests is crucial.

The diagnosis of toxoplasmosis is commonly performed using serological assays. The first laboratory test developed for *Toxoplasma* antibodies was the dye test;[2] since then, many other methods have been introduced.

Since a relatively high percentage of people have anti–*Toxoplasma* antibodies without active infection, more than one serodiagnostic test is required to differentiate patients with active infection and those with persisting antibodies from a past infection. The presence of specific IgM antibodies is considered an acute-phase marker. However, the available assays have certain drawbacks, such as the risk of false positive results due to anti-nuclear antibodies or rheumatoid factor.[3] Moreover, IgM antibodies are not generated in all cases of acute toxoplasmosis and antibody production may persist for more than a year after the initial infection.[4] Thus, reliance on IgM as an acute-phase marker may not be practical.[5–9]

Several components of *T. gondii* have been evaluated as possible diagnostic tools. Previous studies have demonstrated a cluster of *Toxoplasma* antigens in the range of 26–38 kDa that can be detected by Western blotting of cerebrospinal fluids and sera from patients during toxoplasmosis.[10] Antigenic components of 28–36 kDa of *T. gondii* were also reported to be recognized by human IgM antibodies in serum samples from patients with acute toxoplasmosis.[11,12] Among these proteins is the major surface protein of the parasite, SAG1 (p30), which is one of the most immunogenic *T. gondii* antigens. It is highly abundant and expressed only on rapidly dividing tachyzoites, but not on bradyzoites, which grow slowly during the chronic infection stage.[13] Recently, increasing attention has been directed towards direct detection of *Toxoplasma* antigens in the serum samples of infected patients.[14,15]

In the development of an antigen detection test, it is important to have a panel of serum samples that have been shown to have circulating antigen. Thus, in this study, we developed an ELISA that will help identify serum samples with circulating antigens, these can then form part of the panel of positive serum samples for the development of the antigen detection assay. Other than increasing the chance for successful development of such an assay, the antigen-positive serum panel will also allow studies on sequence characterization of the *T. gondii* circulating antigens.

Previously, a study was conducted to describe an ELISA system for detection of soluble *T. gondii* (p30) antigen in human cerebrospinal fluid (CSF) samples from HIV seropositive patients.[16] The results showed a positive detection rate of 45% (119/263). Thus, we have applied this format to develop an ELISA to detect circulating *T. gondii* antigen in human serum samples. Furthermore, we used Western blot analysis in the identification of antigenic proteins in the samples.

EXPERIMENTAL

Serum Samples

Based on clinical information and laboratory diagnosis for toxoplasmosis, a total of 324 serum samples were used in this study. Samples were obtained from serum banks at several institutions in Malaysia (Universiti Sains Malaysia, Universiti Kebangsaan Malaysia, and Universiti Malaya), and were collected according to the ethical requirements of each organization. Two serological assays were employed in the laboratory diagnosis, namely the Platelia Toxo IgM TMB kit for IgM antibody (Bio-Rad, France) and a laboratory-based indirect IgG-ELISA. The cut-off OD of the latter is 0.52, derived from mean OD + 2SD of 44 samples of individuals tested negative by Platelia Toxo IgG kit. From these tests, the serum samples were classified as follows: Group I (probable acute infection): IgM + IgG + or IgM + IgG-, n = 75; Group II (chronic infection): IgM-IgG+, n = 206; Group III (normal serum): IgM-IgG-, n = 30. Sera from patients with other parasitic infections (Group IV) were also included in this study (n = 13).

Toxoplasma gondii IgG avidity EIA (Labsystems, Finland) was performed on a random slection of nine serum samples from Group I, i.e., maximum number of samples that can be tested per kit. Three samples were found to be of low avidity, one borderline, one had high avidity, while four samples needed restesting using higher serum dilution. We were unable to perform further testing using the kit due to its high cost.

Preparation of Soluble *T. gondii* Protein

T. gondii (RH strain) was grown intraperitoneally in Balb/c mice for 3 to 4 days. The peritoneal exudate was obtained and washed three times by centrifugation at 3000 rpm (Eppendorf, 5415R, Handburg, Germany) for 10 minutes with phosphate-buffered saline (PBS, pH 7.2). The tachyzoites were then sonicated for 6 times (30 seconds each time), and the homogenate was centrifuged at 10,000 g for 15 minutes to remove cell debris and stored at −70°C until use.

Preparation of *T. gondii* SAG1 Recombinant Proteins

The SAG1 DNA sequence (GenBank Accession No. S76248) was amplified as described previously,[17] with some modifications. PCR-cloning was performed using *T. gondii* DNA and the following primers: TGP30F (5′-GAC GAG TAT GTT TCC GAA GGC AGT GAG ACG-3′) and TGP30R (5′-AGC CGA TTT TGC TGA CCC TGC AGC CC-3′). The PCR product was sequenced to confirm absence of mutation, cloned into TOPO cloning vector (Invitrogen, USA), then subcloned into pPROEX-HT (Life Technologies, Gaithersburg, MD) expression vector. A final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce protein expression of the recombinant bacterial culture at $OD_{600\,nm}$ 0.5, and harvested at 4 hours post-induction.

After cell breakage using a French press, it was affinity-purified using Ni-NTA resin (Qiagen, Germany). To confirm the presence of the 37.5 kDa histidine-tagged recombinant protein, Western blot analysis was performed using anti-His conjugated to peroxidase (HRP) antibody (Qiagen, Germany). In order to obtain highly purified protein, the affinity-purified recombinant protein was electrophoresed and the correct band was electro-eluted, followed by concentration and buffer exchange into PBS, pH 7.2.

Preparation of Polyclonal Rabbit Anti-*Toxoplasma* IgG Antibodies

Polyclonal rabbit sera from animals hyperimmunized with *T. gondii* extract were previously produced in the laboratory of one of the authors. Purification of IgG antibody from rabbit serum was performed by using a commercial spin-column (Melon™ Gel IgG purification kit; Pierce, USA). Briefly, serum samples were diluted 1:10 with one time Melon™ Gel Purification Buffer. A volume of buffer 10 times the gel bed volume was added and allowed to flow through the column. The diluted serum sample was added and allowed to flow through the column. The diluted serum sample was added and 0.5–1.0 mL of the IgG fraction was collected. The absorbance of each fraction was measured at 280 nm using a biophotometer (Eppendorf, USA). Protein-containing fractions were pooled and concentrated three times using a spin column with a 3 kDa molecular weight cut-off.

Circulating Antigen Detection ELISA (CA-ELISA)

The format for development of the assay was modified from a method described previously.[16] Other than the use of a different kind of patients' samples (i.e., serum instead of CSF), the diluent and reagents used in the other layers of the ELISA sandwich were also different from the previously

reported assay. In this study, rabbit anti-*Toxoplasma* IgG (instead of biotinylated mouse anti-*Toxoplamsa* IgG) followed by goat anti-rabbit IgG –HRP (instead of avidin-HRP), and finally ABTS (instead of H_2O_2/ o-phenylenediamine) was used as substrate.

Monoclonal anti-SAG1 (p30) antibody (BiosPacific, USA) was diluted with 0.1 M bicarbonate buffer (pH 9.6) to a concentration of 10 μg/mL. Each well of the polystyrene microtiter plate (Maxisorp™; Nunc, Denmark) was filled with 50 μL of this reagent and incubated overnight at 4°C. The wells were then washed 5 times (5 minutes each) with PBS containing 0.05% Tween 20 (PBS-T). The wells were then blocked with 100 μL of 1.0% (w/v) BSA in PBS-T and incubated for 1 hour at 37°C, followed by a washing step as described above. Then, 50 μL of the serum samples, and positive and negative control sera (1:200 dilutions in 1% BSA in PBS-T) were incubated for 2 hours at 37°C. The negative control serum was comprised of 30 normal serum samples pooled together. To generate a positive control, 200 μL of normal serum was spiked with 0.25 mg of crude soluble *Toxoplasma* protein. After a washing step, 50 μL/well of rabbit polyclonal anti-*T. gondii* IgG antibody was diluted 1:12,000 with 1.0% (w/v) BSA in PBS-T. The plate was incubated for 1 hour at 37°C and then washed. Then, 50 μL/well of goat-anti-rabbit IgG conjugated to horseradish peroxidase (KPL, USA) was diluted 1:500 with 1% (w/v) BSA in PBS-T and incubated for 1 hour, followed by another washing step. Finally, 50 uL/well of ABTS substrate solution for horseradish peroxidase (Roche Diagnostic, Germany) was added and the reaction was further incubated for 30 minutes at 37°C. The absorbance of each reaction mixture was determined at 405 nm with an ELISA microplate reader (Tecan Sunrise, Austria). The cut-off value (COV) of the antigen detection ELISA was calculated as an O.D. value of 0.12 which was derived from the mean optical densities (OD) of 30 normal serum samples plus two standard deviations.

Antigen Detectable Limit by CA-ELISA

The detectable limit of the antigen detection assay was determined by spiking pooled negative serum sample with serially diluted *T. gondii* soluble and SAG1 recombinant proteins followed by perfoming the CA-ELISA.

Depletion of Major Serum Proteins

To analyze *T. gondii* circulating antigens in serum samples by Western blotting, depletion of major serum proteins was carried out using ProteoPrep20 Plasma Immunodepletion Kit (Sigma, U.K.). This method was applied to remove extremely abundant protein from serum samples that may interfere

with the detection of proteins with low abundance in the serum samples. Based on the results of the antigen detection ELISA, depletions were performed on pooled postive and negative serum samples, each from 10 individuals.

SDS-PAGE and Western Blot Analysis

The depleted antigen-positive serum samples from normal, active, and chronic toxoplasmosis patients were pooled separately and subjected to electrophoresis (10% SDS-PAGE). The protein bands were transferred onto a nitrocellulose membrane (Osmonic, GE) using a semi-dry TransBlot electroblotting system (Bio-Rad, USA) for 30 minutes at 12 V. The membrane was then cut into strips, blocked with 1% casein solution (Roche Diagnostic) for 1 hour and then probed overnight with primary antibodies, which consisted of non-depleted pooled antigen-positive serum samples from active, chronic or normal patient samples. These serum samples were diluted 1:20 with 0.5% casein solution (Roche Diagnostic). This was followed by a 1 hour incubation with the appropriate antibody conjugates, namely mouse anti-human IgM conjugated to horseradish peroxidase (Zymed, USA) or mouse anti-human IgG (Zymed, USA) conjugated to horseradish peroxidase, at a dilution of 1:1000 and 1:2000, respectively. The washing solution used in-between incubation steps was TBS with 0.05% (v/v) Tween 20. Finally, the membrane was developed by using BM chemiluminescence blotting substrate (Roche Diagnosrtic, Germany) and Kodak films (Kodak, USA).

Statistical Analysis

A Z test for two population proportion was used to compare the percentage of positive cases detected in active and chronic infection. A p value of 0.01 or less was considered to be significant.

RESULTS

Detection of Circulating *Toxoplasma* Antigen in Serum Samples Using ELISA

The results of the double sandwich ELISA to detect circulating antigens in toxoplasmosis serum samples are shown in Table 1. Twenty one out of 75 serum samples (28%) from probable active toxoplasmosis cases were positive by the assay. The assay also detected circulating antigens in 11% of individuals with chronic toxoplasmosis (23/206). Statistical analysis indicated a significant difference ($p < 0.01$) between the two kinds of samples. No false positive results were obtained when the assay was tested with sera from 43 serum samples from normal individuals and patients with

TABLE 1 Detection of Circulating *Toxoplasma* Antigen in 324 Sera of Infected and Non-Infected Individuals Using CA-ELISA

Status of *T. gondii* Infection[a]	SAG-1 (p30) Antigen Detection ELISA			
	No.	Positive	Negative	% Positive
Toxoplasma infected individuals COV ≥ 0.12				
Acutive infection	75	21	54	28[b]
Chronic infection	206	23	183	11[b]
Non-infected individuals (IgG and IgM negative for toxoplasmosis)				
Normal individual	30	0	30	0
Other parasitic infection	13	0	13	0
Total	43	0	43	100

[a]Based on clinical examination and combined evaluation of anti-*Toxoplasma* IgM and IgG antibodies using ELISA.

[b]Significant differences ($p < 0.01$) were observed between positive rates in active and chronic infections as calculated by Z test for two population proportion.

other parasitic infections, including amoebiasis (n = 4), soil transmitted helminthiasis (STH) (n = 3), toxocariasis (n = 2), or filariasis (n = 4).

Antigen Detectable Limit by CA-ELISA

As shown in Figure 1, *Toxoplasma* soluble and SAG1 recombinant proteins gave the lowest detection limit of CA-ELISA of 31.25 ng/mL and 62.50 ng/mL respectively.

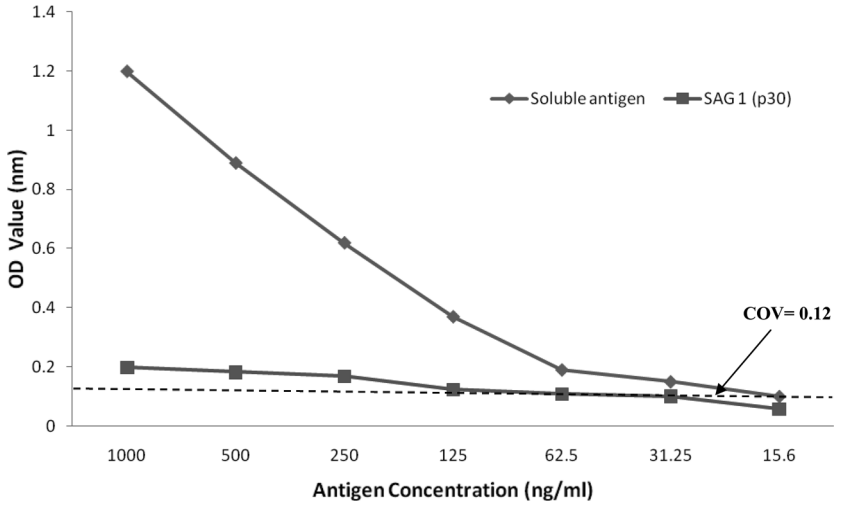

FIGURE 1 Detection limit of the CA-ELISA using *T. gondii* soluble and SAG1 recombinant proteins in spiked normal serum.

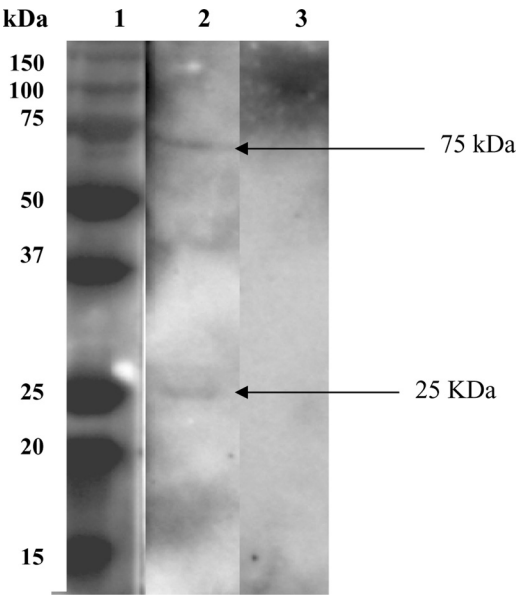

FIGURE 2 Westerns blot of the depleted serum samples from probable active cases. Lane 1: Unstained marker; Lane 2: Immunoblot performed using pooled sera from positive patients (IgM+, IgG+, CA+); Lane 3: Immunoblot performed using pooled sera from normal individuals (IgM-, IgG-, CA-).

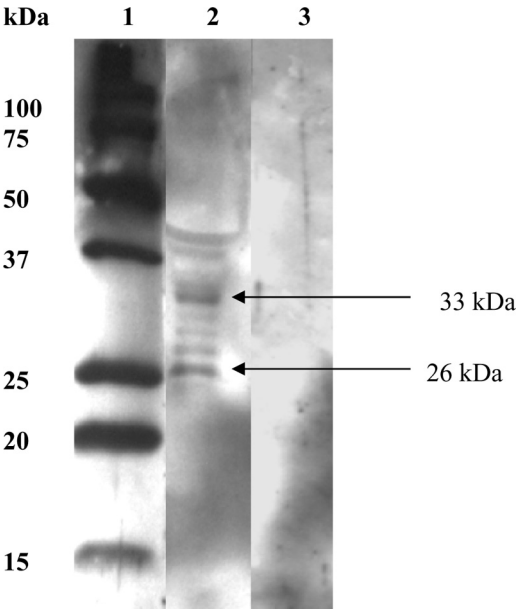

FIGURE 3 Westerns blot of the depleted serum samples from probable chronic cases. Lane 1: Unstained marker; Lane 2: Immunoblot performed using pooled sera from positive patients (IgM-, IgG+, CA+); Lane 3: Immunoblot performed using pooled sera from normal individuals (IgM-, IgG-, CA-).

Detection of Circulating *Toxoplasma* Antigen in Sera of Infected and Non-Infected Individuals Using Western Blot

SDS-PAGE and Western blot analysis of depleted pooled sera positive for circulating antigen from probable active and chronic toxoplasmosis patients was carried out to investigate the antigenicity of the proteins in the samples (Figs. 2 and 3). In the active infection sample, two bands with molecular weights of 75 and 25 kDa showed reactivity, while five bands ranging in molecular weight from 26 kDa to 33 kDa were identified in the chronic infection sera. No bands were found in the Western blot of normal sera.

DISCUSSION

Demonstrating the presence of proteins from pathogenic organisms in sera or other body fluids of patients with an acute/active infection is becoming an important diagnostic tool, especially in patients with impaired immune responses for whom a rapid and accurate diagnosis is critical. Considerable tissue and parasite destruction occurs during the active infection of *T. gondii* in humans. It is therefore plausible that *Toxoplasma* antigen is present in the blood for a short period of time, either in a free form or in immune complexes.[18] The presence of circulating *T. gondii* antigens in infected humans beings has been investigated and considered as an important indicator of active infection.[4,14,19–21]

In this study, we have developed an ELISA for detection of circulating *Toxoplasma* antigen (CA-ELISA) in patient serum by employing monoclonal anti-SAG-1 (p30) as the capture antibody. This method was modified from a previous study,[16] which detected soluble *Toxoplasma* antigen in human cerebrospinal fluid (CSF). Monoclonal antibodies of defined specificity are a powerful tool for serological detection of antigens, and false-positive results due to rheumatoid factor was reported not to occur when monoclonal antibodies were used in the assay.[22] In previous studies that used polyclonal antibodies in ELISAs to detect circulating proteins, a relatively low proportion of positive rates were reported.[4,19] We tried using polyclonal rabbit anti- *T. gondii* IgG as the capture antibody in two different ELISA formats, but our findings (data not shown) showed that the assays were unable to discriminate between positive and negative samples. This may be caused by non-specific binding of antigens to antibodies, since a lot of other interfering proteins may exist in the serum samples or the polyclonal antibodies may be cross-reacting to multiple epitopes.

SAG1 (p30) is the major surface protein of *Toxoplasma*, and is expressed exclusively by tachyzoites, which are the rapidly replicating forms of the parasite. This molecule is involved in parasite attachment[23–24] and could be an important indicator of active infection.[4,25] In the present study,

detection of circulating *Toxoplasma* antigen was achieved using a capture-ELISA which employ a microtitre plate coated with *Toxoplasma* SAG1 monoclonal antibody. Serum samples which gave positive results will be expected to contain *Toxoplasma* SAG1 protein, thus these samples will also be expected to contain other circulating antigens. The CA-ELISA method detected 31.25 ng/mL and 62.50 ng/mL of *T. gondii* soluble and SAG1 recombinant proteins respectively. In a previous study which employed soluble *Toxoplasma* antigen, the same detection limit (31.25 ng/mL) was reported.[19]

This study showed the presence of circulating antigens in 28% (21/75) of serum samples from patients with probable active infection, whereas circulating antigens were detected in 11% (23/206) of serum samples from patients with probable chronic infection were positive. There was a statistically significant difference observed between these groups ($p < 0.01$) indicating that detection of SAG1 (p30) protein has the potential in detecting active infection. Our results indicated a lower detection rate compared with previous findings[16] which reported positive rate of 45% (45/263). This is probably due to the difference in the kind of sample employed which may influence the sensitivity of the assay, namely serum in our case versus CSF in their case. Similar to our results, previous investigators who employed serum samples also observed low detection rates of circulating antigen in active toxoplasmosis cases, with 5.7% (64/1116) and 30% (13/42).[4,20] In addition, this is also in agreement with another finding,[18] which reported that a low percentage of circulating antigen [0.22% (8/3598) to 2.11% (7/322)] was observed in the sera of patients who are suspected of having toxoplasmosis. The low detection rate reported thus far may show that circulating *Toxoplasma* antigens do not circulate for long periods of time as compared to specific IgM antibodies.[4,20,26] On the other hand it also shows that more work is still needed in the development of a highly sensitive antigen detection assay for toxoplasmosis.

Chronic toxoplasmosis is charaterized by infection immunity or premunition.[27] Effective immunity is probably maintained and boosted by relapsing infections, such as ruptured tissue cysts,[28] and steadily increasing liberation of antigenic materials from the infected organ and cells into the circulation.[29] The existence of circulating antigens in chronic cases in this study and other studies suggested an exacerbation of an old infection or reinfection.[4] A high IgG antibody titer in chronic cases may cause immunocomplex formation with the larger part of the soluble *T. gondii* antigen in the serum,[20] thus leading to the positive results observed in the assay.

Remarkably, the CA-ELISA in this study showed no false positive results or cross-reactivity when tested with sera from normal patients and patients with other parasitic infections. It can thus be concluded that the CA-ELISA

allows for differentiation between patients with or without *Toxoplasma* infection. As far as we know, the specificity rate of 100% (43/43) is the highest documented thus far, with the highest previous specificity rate at 91% (29/32).[14]

In the development of a serological assay, a panel of 'true' positive and negative serum samples are needed. In the case of acute toxoplasmosis, true positive samples are usually those with high IgM antibodies and low IgG avidity titres, in combination with consistent clinical symptoms of the patients from whom the samples were taken from. However, the cost for testing for IgG avidity using commercial kits is prohibitive, especially for non-developed countries. Thus the use of the CA-ELISA introduced in this study will help as an additional criteria in the initial important step of selecting a panel of serum samples for use in developing a highly sensitive antigen detection assay for active toxoplasmosis.

To date, only a few studies have been performed to characterize the circulating antigens of *T. gondii* in human infection. In one study, sera containing circulating immune complexes from children with congenital toxoplasmosis and from pregnant women with IgM antibodies to *Toxoplasma* were found to demonstrate 55–58, 48, 44, 38, 30 and 26 kDa antigenic components.[30] In other studies, IgM-positive sera from active toxoplasmosis patients recognized *Toxoplasma* proteins that were 36 and 60 kDa, while a 12 kDa protein was observed in the chronic phase.[21,22] In our current study, we found an obvious difference in the sizes of antigenic protein bands between active and chronic infection sera. Two antigenic bands with molecular weights of 25 and 75 kDa were detected in the pooled active sera, whereas five antigenic protein bands were present in the pooled chronic sera, ranging in size from 26 to 33 kDa. The latter finding is similar to a previous study which reported the presence of 26–38 kDa antigenic bands in CSF and serum of toxoplasmosis patients.[10] None of these bands were recognized by normal serum samples. However, it is interesting to note that the SAG1 protein (30 kDa) was not observed in the Western blots of the pooled active sera. One possible explanation is that in this sera group, one or more of the abundant human serum proteins may have been differentially expressed and bound to the p30 antigen, thus the latter may be lost when the sample was ran through the immunodepletion colum prior to electrophoresis of the sample.

CONCLUSION

In conclusion, the CA-ELISA developed in this study will be useful for selecting serum samples containing *T. gondii* antigens for use in the development of a sensitive and specific antigen detection test for active toxoplasmosis

and for characterization studies of the circulating *T. gondii* antigens by Western blots and mass-spectrometry.

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