Original Article

Novel IMB-ELISA Assay for Rapid Diagnosis of Human Toxoplasmosis Using SAG1 Antigen

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SUMMARY: Nanotechnology is important for medical diagnosis. Various nanoparticles have presented tremendous potential for diagnosing disease markers, pre-cancerous cells, fragment of viruses, specific proteins, antibodies, and other disease indicators. In general, nanoparticles are smaller than 1,000 nm and produced from different materials in different shapes such as spheres, rods, wires, and tubes. Our study aimed to develop a novel antigen-capture immunoassay based on IgG polyclonal antibody-coated magnetic microbead nanoparticles for the rapid detection of circulating surface antigen 1 of Toxoplasma gondii in human serum samples. Sandwich ELISA elicited a sensitivity of 92%, a specificity of 92.7%, a positive predictive value (PPV) of 92%, and a negative predictive value (NPV) of 92.7%. Immunomagnetic bead-ELISA showed sensitivity (98%), specificity (96.4%), PPV (96%), and NPV (98.1%) higher than that of sandwich ELISA. It is obvious that the use of magnetic microbead nanoparticles offers the potential advantage of improving the diagnostic testing of toxoplasmosis.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite with an extremely wide geographic and host range (1). The course, symptoms, and consequences of T. gondii infection depend on the virulence of the parasite as well as on the genetic background and immune status of the infected host (2). It is estimated that one third of the world’s population has been infected (3). High prevalence of the infection has been reported among pregnant women and women of childbearing age in Latin America, Eastern/Central Europe, Middle East, south-east Asia, and Africa (4).

Diagnosis of T. gondii infection relies mainly on serological tests enabling the detection of specific IgG, IgM, and IgA anti-Toxoplasma antibodies. Detection of IgM and IgA suggests an active T. gondii infection, whereas IgG alone leads to the diagnosis of a chronic infection. However, conflicting results may arise since anti-T. gondii IgM antibodies can be detected for more than a year after the initial infection (5).

A major emphasis of medical technology is to improve diagnostic techniques to screen for infectious diseases. Such screening is required to identify illnesses, assess risk factors for disease onset, and determine progression or improvement of disease state. Especially, nanotechnology may improve the sensitivity, selectivity, speed, cost, and convenience of diagnostic tests. Furthermore, nanotechnology has also opened up the possibility of other screening strategies.

Attachment of T. gondii to host cells involves surface antigens of the parasite, which are recognized as major study targets. There are 5 proteins in the superfAMILY of the surface antigens (SAG) of T. gondii, SAG1, SAG2 (22 kDa), p23, p35, and SAG3 (43 kDa). SAG1 is a 30 kDa glycoprotein designated as SAG1/p30 (6); it is stage specific, detected only in the tachyzoite stage, and absent in the bradyzoite stage (6,7). This antigen is abundant on the surface of both extracellular and intracellular tachyzoites (6). It has also been reported that SAG1 can elicit a lethal inflammatory process in a mouse model of pathogen-driven ileitis (8); however, the biological role of this surface protein remains unclear. SAG1 is considered as an important candidate antigen in diagnostic tests, with no known cross-reactivity with proteins from other microorganisms (6).

This work aimed to evaluate SAG1 antigen as an early diagnostic marker of toxoplasmosis. A novel nanodiagnostic assay, immunomagnetic bead (IMB)-ELISA, based on IgG polyclonal antibody (pAb) was developed to detect circulating SAG1 antigens in human sera. Exploring an effective early diagnosis might be a key step towards prevention and eradication of this disease.

MATERIALS AND METHODS

Participants: This study enrolled outpatients of the Theodor Bilharz Research Institute (TBRI) and ELkasrElainy Hospital. All patients and healthy volunteers were subjected to a panel of tests (according to the Toxoplasma Serological Profile [TSP]), consisting of the latex agglutination test, an ELISA based on crude T. gondii tachyzoite lysate (9), the Sabin-Feldman dye test (10), a double sandwich IgM ELISA, and the Differential agglutination test (Cofacetone [AC]-fixed versus that of formalin [HS]-fixed tachyzoites) (AC/HS test). These tests have been used successfully to determine if infections were acquired in the recent or more distant past. Ethical issues were strictly handled according to the International Ethical Guideline for Biomedical
Research. Prior to blood collection, the purpose of the study was explained individually to all participants and informed consents obtained. According to TSP, participants were divided into 3 main groups: (i) Fifty T. gondii-infected patients selected based on the presence of antibodies in their serum. (ii) Thirty-five patients harboring other parasites (E. histolytica, n = 15; Giardia, n = 5; and Cryptosporidium, n = 15). (iii) Twenty parasite-free healthy individuals from the TBRI medical staff served as negative control.

Serum samples from all groups were tested to detect circulating toxoplasmal antigens. Sera were separated and stored at −70°C until required.

**Animals:** New Zealand white rabbits, weighting approximately 1.5 kg and 6 weeks old, were obtained and housed in TBRI. They were kept for 4 weeks (experiment duration) under standard laboratory care, i.e., 21°C, 16% moisture, filtered drinking water, and 15% protein, 3% fat, and 22% fiber diet. Blood samples were collected from healthy rabbits before inoculation and examined by ELISA for T. gondii antibodies and cross reactivity with other parasites, as previously described (16). Animal experiments were carried out according to the international guidelines and TBRI animal ethics committee.

**Preparation of tachyzoite proteins (SAG1 antigen) of T. gondii:** T. gondii antigens were prepared from peritoneal exudates of BALB/c mice infected 3 days earlier with tachyzoites of T. gondii RH strain, as previously described (11). Mice peritoneal exudates were centrifuged at 2,000 g for 20 min, washed 3 times with phosphate buffer saline (PBS), subjected to sonication periods, and centrifuged at 12,000 g for 1 h, and supernatants were collected as soluble antigens. Protein content was determined by the Bradford method, and protein content was measured according to Bradford (21). The reactivity of anti-T. gondii IgG pAb against SAG1 antigen was measured by indirect ELISA with some modifications from the original method of Engvall and Perlman (15). Labeling of anti-SAG1 IgG pAb with horseradish peroxidase with the periodate method was performed as previously described (22).

**Sandwich ELISA:** Polystyrene microtiter plates were coated with 100 μl/well of purified IgG pAb diluted in 8 ml carbonate buffer, pH 9.6. The plates were washed 3 times with a washing buffer (0.1 M PBS/Tween, PH 7.4/Tween). The free sites were blocked with 200 μl/well of 0.1% BSA/0.1 M PBS/T, pH 7.4 and incubated for 1 h at 37°C. The plates were washed 5 times with 0.1 M PBS/T. Sera (100 μl/well) were added and incubated for 2 h at 37°C, and the plates were washed 3 times. Peroxidase-conjugate IgG pAbs (100 μl/well) diluted in PBS were dispensed, and plates were incubated for 2 h at 37°C. The reaction was visualized by the addition of 100 μl/well of O-phenylenediamine substrate solution for 30 min in the dark at room temperature. The reaction was stopped by adding 50 μl/well of 3 N H₂SO₄ and the plates were read at 492 nm with an ELISA microplate reader (Bio Rad).

**IMB-ELISA:** Microtitration plates were coated with 100 μl/well of anti-T. gondii IgG pAbs coupled with magnetic microbead nanoparticles (200 μg/ml for IgG in 8 ml carbonate buffer, pH 9.6) and incubated overnight at room temperature. Plates were washed 3 times with 0.1 M PBS/T, pH 7.4. The remaining sites were blocked by 100 μl/well of 2.5% FCS/PBS/T and incubated for 2 h at 37°C. The plates were washed 3 times with PBS/T. Serum samples (100 μl) were pipetted into the wells in duplicate and incubated for 2 h at 37°C. The wells were washed 3 times, and 100 μl/well of peroxidase-conjugated pAbs were added and incubated for 1 h at room temperature. The reaction was visualized as described above.

**Statistical analysis:** Data are expressed as mean ± standard deviation (SD) or percentages (%). The SPSS software (version 13 Windows) was used for data analysis.

**RESULTS**

**Purification of 30 kDa SAG1 of T. gondii:** (i) DEAE-Sephadex G-50- ion exchange chromatography: Fig. 1 shows the OD₅₀₀ profile of the antigen fractions obtained following purification by DEAE Sephadex G-50 ion exchange chromatography. The eluted antigens were represented by 4 peaks at 12, 22, 25, and 34 with a maximum OD value of 0.068 for fraction 25. The eluted protein fractions resulted from this purification method were analyzed by 12.5% SDS-PAGE under reducing condition and showed 3 major bands at 80, 30, and 27 kDa and many minor bands ranging from 110 kDa down to 17 kDa. (ii) DEAE-Sephadex G-200- ion ex-
change chromatography: Fig. 2 shows the OD$_{280}$ profile of the antigen fractions obtained following purification by DEAE Sephadex G-200 ion exchange chromatography. The eluted antigen was represented by a single peak with a maximum OD value of 0.15 for fraction 23.

The eluted protein fraction was analyzed by 12.5% SDS-PAGE under reducing condition and showed a unique band at 30 kDa, which represents 30 kDa SAG1 (Fig. 3).

Estimation of total protein content of *T. gondii* antigens: The protein content was 7 mg/ml of crude antigens and 3.5 and 1.5 mg/ml of purified antigens after DEAE-Sephadex G-50 and G-200 gel filtration chromatography, respectively.

(i) Reactivity of SAG1 antigen by indirect ELISA: The antigenicity of the purified antigen was tested by an indirect ELISA technique. Blood samples from human-infected with *T. gondii* elicited a strong reaction against 30 kDa SAG1 with a mean OD reading of 1.841, and no cross reactions were detected in samples of patients infected with other parasites (Table 1).

Production of pAbs against SAG1: Test blood samples were withdrawn from New Zealand white rabbits before the injection of each immunizing dose. They were tested for the presence of specific anti-*T. gondii* antibodies by indirect ELISA, and antibody levels increased 1 week after the first booster dose. Three days after the second booster dose, immune sera displayed high titers against 30 kDa SAG1 with maximal OD of 1.5 at 1/100 dilution. The total protein content in the crude rabbit serum containing anti-*T. gondii* pAbs was 12.5 mg/ml.

After 50% ammonium sulfate precipitation, the protein content was 8 mg/ml, while it dropped to 4 mg/ml following 7% caprylic acid precipitation. The purity of IgG pAbs after each purification steps was assayed by 12.5% SDS-PAGE under reducing condition. The purified IgG pAbs were represented by H- and L-chain bands at 53 and 31 kDa, respectively. The pAbs appear free from other proteins (Fig. 4).

The reactivity of anti-*T. gondii* IgG pAbs against SAG1 and other parasite antigens was determined by indirect ELISA. The produced anti-*T. gondii* pAbs diluted 1/100 in PBS/T buffer elicited a strong reactivity towards SAG1. The OD reading at 492 nm for *T. gondii* antigen was 2.79 compared to 0.532, 0.326, and 0.243 for *E. histolytica*, *Giardia*, and *Cryptosporidium*, respectively.

Table 1. The reactivity of SAG1 antigen by indirect ELISA

<table>
<thead>
<tr>
<th>Parasitic antigen</th>
<th>OD reading Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. gondii</em></td>
<td>1.841 ± 0.231</td>
</tr>
<tr>
<td><em>E. histolytica</em></td>
<td>0.294 ± 0.221</td>
</tr>
<tr>
<td><em>Giardia</em></td>
<td>0.126 ± 0.049</td>
</tr>
<tr>
<td><em>Cryptosporidium</em></td>
<td>0.142 ± 0.082</td>
</tr>
</tbody>
</table>

Standardization of sandwich ELISA: The optimum dilution of anti-*T. gondii* IgG pAb for the coating step was 1/80, while it was only 1/40 for peroxidase-conjugated anti-*T. gondii* IgG pAb.

Detection of circulating *T. gondii* SAG antigen in human serum samples: (i) Sandwich ELISA: To measure the incidence of positivity for *T. gondii*, it was necessary at first to determine the cut off value for posi-
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**Table 2. Detection of 30 kDa SAG1 in human sera samples using sandwich ELISA**

<table>
<thead>
<tr>
<th>Group (no. of animals)</th>
<th>Positive case</th>
<th>Negative case</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. X ± SD</td>
<td>No. X ± SD</td>
<td></td>
</tr>
<tr>
<td>Healthy control (n = 20)</td>
<td>20 0.21 ± 0.07</td>
<td>20 0.12 ± 0.07</td>
</tr>
<tr>
<td>T. gondii (n = 50)</td>
<td>49 1.94 ± 0.32</td>
<td>15 0.22 ± 0.032</td>
</tr>
<tr>
<td>E. Histolytica (n = 15)</td>
<td>11 0.49 ± 0.21</td>
<td>14 0.24 ± 0.16</td>
</tr>
<tr>
<td>Giardia (n = 5)</td>
<td>0 —</td>
<td>5 0.20 ± 0.09</td>
</tr>
<tr>
<td>Cryptosporidium (n = 15)</td>
<td>21 0.82 ± 0.14</td>
<td>14 0.14 ± 0.10</td>
</tr>
</tbody>
</table>

*a,*: See Table 2, footnote.

**Table 3. Detection of 30 kDa SAG1 in human sera samples using IMB-ELISA technique**

<table>
<thead>
<tr>
<th>Technique</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>92</td>
<td>92.7</td>
<td>92</td>
<td>92.7</td>
</tr>
<tr>
<td>IMB ELISA</td>
<td>98</td>
<td>96.4</td>
<td>96</td>
<td>98.1</td>
</tr>
</tbody>
</table>

**Table 4. The sensitivity, specificity, PPV and NPV of sandwich and IMB-ELISA results used for detection of 30 kDa SAG1 in human sera samples**

**DISCUSSION**

Infections by *T. gondii* are widely prevalent in humans and animals, especially in food animals throughout the world (23). Early diagnosis is important in patients who present clinical symptoms of toxoplasmosis and in pregnant women to prevent severe congenital infection in the developing fetus (24,25). Toxoplasmosis diagnosis depends on direct and indirect methods. The classical diagnosis of toxoplasmosis relies on serological methods for the detection of specific immunoglobulin antibodies. However, these methods have a poor efficiency, especially in neonates and immunodeficient patients (26). Owing to IgM long half-life in many patients and high percentages of non-specific results, toxoplasma-specific IgM assay is no longer of benefit in diagnosing acute toxoplasma infection (27–29).

In some studies years ago, *T. gondii* antigens present in serum samples of experimentally infected animals suggests that antigens are detectable during the acute phase of toxoplasmosis (30,31). For instance, Raizman and Neva (32) showed the presence of circulating antigens in mouse sera by counter-current electrophoresis and agar gel diffusion on days 2–4 of infection. Huskinson et al. (33) could demonstrate the presence of *T. gondii* antigens in serum and urine of mice as early as 5 days post infection by western blot. Recently, Shojaee et al. (34) detected antigenemia from the fourth day after infection in mouse sera by immunoblotting. Moreover, to
increase the diagnostic potency of antigens, isolation of their immunogenic fractions could be useful (35). Tachyzoites are thought to be responsible for acute phase of the infection and express immunodominant antigens that induce strong immune responses (36). Abdel-Rahman et al. (37) used *T. gondii* crude and affinity-purified tachyzoite antigens isolated from slaughtered sheep in the diagnosis of toxoplasmosis in horses. Conde de Felipe et al. (38) proved that 29–35 kDa *T. gondii* fractions can detect specific IgG in goats 2 weeks earlier than crude extract. The 30 kDa surface protein of *T. gondii* tachyzoite represents 3% to 5% of the parasite total proteins (39) (Kasper, 1987) and has been proposed for the diagnosis of acute acquired toxoplasmosis. Cesbron et al. (40) and Santaro et al. (41) have developed a double-sandwich ELISA based on this protein for detection of IgM antibodies.

SAG1 is the main surface antigen of *T. gondii* that induces high titers of IgG, IgM, and IgA despite only accounting for 3–5% of total cellular proteins (42). In addition, this protein is also a tachyzoite-specific antigen and is highly conserved (43); thus, it is important for diagnostic purposes (44,45). Tachyzoites are the key disease-causing forms. During the acute stage of infection, tachyzoites activate a potent host immune response, which eliminates most of the parasites. The surface of the tachyzoite is the main target of the host immune response. SAG1 is a major surface antigen involved in this process. Although it only accounts for 3–5% of *T. gondii* total proteins, the majority of the antibodies are reactive against SAG1 during infection (46). Various SAG1 preparations purified from the parasite (47,48), produced by recombinant systems in *Escherichia coli* (49) or yeast (50), and its constituent peptides (51–53) stimulate the host humoral and cellular immunity, thereby providing protection against toxoplasmosis (54). As such, SAG1 features excellent antigenicity and immunogenicity and is valuable for both diagnosis and immunization. In 1980, Handman et al. (55) first identified SAG1 from *T. gondii* surface membrane antigens using a monoclonal antibody technique. Subsequently, Kasper et al. (56) applied mAb-affinity chromatography to isolate the protein. In 1988, Burg et al. (43) cloned the complete *T. gondii* sag1 gene, which has a length of 1.1 kb, encodes 336 amino acids, and yields a 30 kDa protein.

In this study, we aimed to purify and characterize SAG1 antigen from *T. gondii* tachyzoites and evaluate its early diagnostic potential in human toxoplasmosis with sandwich ELISA and IMB-ELISA techniques, which may aid in the prevention of the infection and eradication of its transmission. When *T. gondii* antigens were analyzed by 12.5% SDS-PAGE under reducing condition, SAG1 antigen was detected as a 30 kDa protein with a concentration of 1.5 mg/ml. These results were similar to that of Villavedra et al. (42,57) who purified and isolated a fraction of 30–33 kDa from crude tachyzoite antigens, which was used successfully in the diagnosis of human toxoplasmosis by ELISA.

The reactivity of SAG1 antigen was tested by indirect ELISA and elicited a strong reaction with a maximal mean OD reading of 1.841. This purified antigen was utilized for immunization of rabbits for the production of pAbs. Which contain the entire antigen-specific anti-body population; thus they offer a statistically relevant idea for the overall picture of an immune response. Our anti-SAG1 IgG pAb was represented by H- and L-chain bands at 53 and 31 kDa, respectively, and a 4 mg/ml protein concentration. These results are comparable with the yield of purified immunoglobulins from any biological fluid following similar purification procedures (58,59). The purified rabbit IgG fraction served as both antigen capture (1/80 μg/ml) and peroxidase-conjugated (1/40 μg/ml) detecting antibody in sandwich ELISA. The cut-off OD value for positivity in sandwich ELISA for SAG1 was 0.428 in serum samples.

Many immunologic tests based on antigen detection (60,61) and molecular or proteomic diagnostic techniques (62,63) have been well studied and described. However, an affordable, easy-to-handle, sensitive, and specific method is not yet available (64). An immunomagnetic bead-based immunoassay is a popular approach in diagnosis of many food-borne and infectious diseases. This innovative technique involves antibody-coated magnetic microbead nanoparticles, which trap antigens from liquid media. Furthermore, the small size and shape of the microbeads enable them to be evenly dispersed within the sample, thus improving the effectiveness of the antibody conjugation, and consequently enhancing the sensitivity of antigen detection (65,66).

In this study, we developed a novel antigen-capture immunoassay based on IgG pAb-coated magnetic microbead nanoparticles for the detection of circulating SAG1 antigens of *T. gondii* in serum samples. The study was conducted on *T. gondii*-infected patients, patients infected with other parasites, and healthy controls. Sandwich ELISA revealed a sensitivity of 92%, specificity of 92.7%, PPV of 92%, and NPV of 92.7%. While IMB-ELISA sensitivity (98%), specificity (96.4%), PPV (96%), and NPV (98.1%) were higher than that of sandwich ELISA.

Our results were comparable to those of Conlan et al. (67), who described the use of IMB as the solid phase for the portable detection of the CSF antigen in spleen samples and for the reliable detection of antibodies against CSF in animals vaccinated with a lapinized C-strain. This IMB-ELISA is 100% sensitive and 91% specific in comparison to polyclonal antibody-based antigen-capture ELISA (AC-ELISA). Moreover, IMB-antibody-ELISA is 97% sensitive and 95% specific. Lei et al. (68) developed a novel egg yolk antibody (IgY)-coated magnetic bead antigen-capture immunoassay for the detection of a circulating antigen of *S. japonica* in serum samples of patients in endemic schistosomiasis areas of China. This IgY-IMB-ELISA comprises IgY pAb-coated magnetic beads as a capture antibody and a monoclonal IgG as a detection antibody. The sensitivity of this magnetic immunoassay is 100% in cases of acute infection and 91.5% in chronic cases of schistosomiasis, and no positive reaction was found in 49 healthy individuals. Cross-reactivity was 3.3% with clonorchiasis and 0% with paragonimiasis. There was a significant correlation between ELISA absorbance values and egg counts (eggs per gram feces) and a correlation coefficient of 0.88 in a small sample of 14 patients. The results demonstrated that IgY-IMB-ELISA is a sensitive and specific assay for detection of *S. japonica*. Ahmed et al. (69) also developed a novel IMB-ELISA based on IgG...
for the detection of excretory/secretory (E/S) antigens in rabbit sera infected with *S. haematobium*. They detected E/S antigens in serum with a sensitivity of 95% and specificity of 93.7% compared to other parasitic infections and of 100% compared to healthy controls. On the other hand, detection of E/S antigens in urine elicited a sensitivity of 91%, and specificity of 93.7% compared to other parasitic infections and of 100% compared to healthy individuals. Furthermore, Aly et al. (70) evaluated a novel magnetic microbead based-latex agglutination assay (MMB-LAT) as a simple test for diagnosis of *S. haematobium* as well as standardizing a novel magnetic microbead based-sandwich ELISA (MMB-S ELISA). They reported that the sensitivity of novel MMB-LAT was 82.4% versus MMB-S ELISA 96.5% and currently used sandwich ELISA 88.2%. The specificity of MMB-LAT was 83.6% versus MMB-S ELISA 96.3% and sandwich ELISA 87.3%.

With our IMB-ELISA results showing a higher sensitivity and specificity than that of sandwich ELISA, it is obvious that the use of magnetic microbead nanoparticles offers the potential advantage of improving the diagnostic testing of toxoplasmosis. The use of magnetic nanoparticles in immunoassay (nanomagnetic assay) combines magnetic nanoparticle high binding capacity and simple separation of bound and unbound materials as a solid phase with the rapid reaction kinetics of solutions, thus providing the chance of enhancing the sensitivity of antigen detection (71–72). The surface of the beads enables substantially more antibodies to be involved in the reaction with the antigen than in sandwich ELISA (73–75).

**Conflict of interest** None to declare.

**REFERENCES**

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