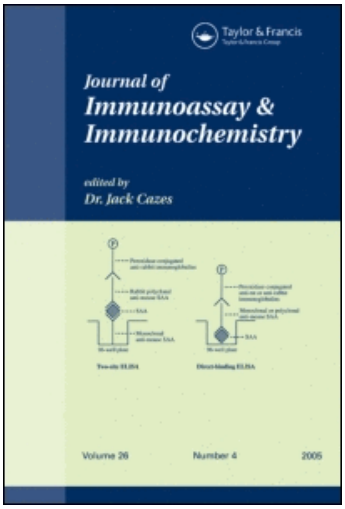

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Immunochemical Identification and Detection of a 36-KDa *Toxoplasma gondii* Circulating Antigen in Sera of Infected Women for Laboratory Diagnosis of Toxoplasmosis

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Abstract: The detection of *Toxoplasma gondii* circulating antigens has been indicated to be a reliable diagnostic approach of active human toxoplasmosis. However, few reports have appeared in the literature regarding the diagnostic potential of *T. gondii* circulating antigens. Here, a specific antibody and western blot analyses were used to demonstrate the presence of a highly reactive antigen of 36-kDa, not only in the extract of *T. gondii* tachyzoites, but also in selected sera of women with confirmed laboratory and clinical signs of recent toxoplasmosis. The 36-kDa *Toxoplasma* antigen

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was purified from *T. gondii* tachyzoites and human serum using electroelution from preparative polyacrylamide gels. The purified polypeptides showed a single peak at 10.9 min when analyzed by capillary zone electrophoresis. Based on the above encouraging results, we have developed an ELISA format for the detection of target *Toxoplasma* antigen (TAg-ELISA) in human serum samples. The TAg-ELISA detected the target antigen in 88% sera of acutely infected women and showed high degree of specificity (91%) among sera from non-infected women. In conclusion, the detection of 36-kDa *Toxoplasma* circulating antigen in human sera appears to be a promising alternative approach for laboratory diagnosis of active *T. gondii* infection.

Keywords: *Toxoplasma gondii*, 36-kDa antigen, Serum, Western blot, ELISA

INTRODUCTION

Infection with *Toxoplasma gondii* occurs worldwide and up to a third of the world's population is currently infected.[1] Acquired toxoplasmosis is usually asymptomatic in most individuals;[2] however, it causes serious morbidity and mortality in fetuses of infected pregnant women[3] and in immunocompromised individuals.[4] The availability of a reliable means of diagnosis early after infection is, therefore, crucial.[5] Various *T. gondii* antigens have been evaluated for their ability to detect *Toxoplasma*-specific IgM antibodies in serum samples of infected patients.[6–8] However, it remains unclear which antigen of the parasite is effective for detection of acute stage-specific IgM antibodies. In addition, IgM antibodies to *Toxoplasma* can be detected for a very long time after the acute phase of infection in some patients.[9,10] Another serodiagnostic marker has been the measurement of the avidity of specific IgG antibodies.[11–13] The usefulness of avidity assays in diagnosing acute toxoplasmosis has been recently demonstrated.[14] However, a low avidity result does not mean the patient had a recently acquired infection[15] and, also, the low avidity result may persist for as long as one year.[5] Ideally, an alternative immunodiagnostic approach should be based on direct detection of *Toxoplasma* circulating antigen using specific antibody.[16,17] Several investigators have reported the direct detection of *Toxoplasma* antigens in serum and urine of experimentally infected animals.[18–21] Preliminary investigations in humans suggested that circulating antigens are only found during acute phase of infection.[22,23] In addition, it was shown that some low molecular weight antigens might be the result of the release of bradyzoites from ruptured tissue cysts and its detection is diagnostic of reactivated toxoplasmosis.[24,25] However, few reports have appeared in the literature regarding the diagnostic potential of *Toxoplasma* circulating antigens in humans.[26–28] The present work was aimed at identifying and characterizing a *Toxoplasma* circulating antigen, as a step in the development of a sensitive and specific immunodiagnostic assay of *Toxoplasma* infection.

EXPERIMENTAL

Serum Samples

Serum samples of 222 women (age range, 18–38 yrs.) attending the outpatient clinic of the Obstetrics & Gynaecology Dept., Tanta University Hospitals, Tanta, Egypt, were included in the present study. General and local clinical examinations were done for all women, including taking thorough histories. Simple stool sedimentation by centrifugation for the detection of parasitic infections was done for each woman. *Toxoplasma* serologic profile (TSP) was determined using a commercially available indirect haemagglutination assay (Toxo-HAI; Laboratoires Fumouze, France) and confirmed using commercially available ELISA kits for anti-*Toxoplasma* IgM or IgG antibodies (ABC Diagnostics, New Damietta, Egypt). On the basis of clinical information and TSP, the women were classified into: no *Toxoplasma* infection (Showing negative results for both specific IgM and IgG antibodies, $n = 32$); acute *Toxoplasma* infection (Showing positive results for specific IgM antibodies either alone, $n = 25$ or in combination with specific IgG antibodies; $n = 121$); and chronic *Toxoplasma* infection (Showing only positive results for specific IgG antibodies, $n = 44$). The IgG seroconversion was confirmed in all 25 women showing only positive results for specific IgM antibodies three months later.

Preparation of Toxoplasma Tachyzoites Antigen

T. gondii (RH strain) was grown intraperitoneally in Balb/c mice for 2 to 3 days. The peritoneal exudate was obtained and washed three times by centrifugation at 4,000 rpm for 10 min with 0.01 M phosphate-buffered saline (PBS, pH 7.2). The tachyzoites were homogenized by repeated freezing at -196°C for 15 min and thawing (3 cycles), then the homogenate was centrifuged at 4,000 rpm for 15 min to remove cell debris. The protein content was then determined according to Lowry et al.[29] and the *Toxoplasma* tachyzoites antigen was stored at -20°C untill used.

Antigenic Preparations of Other Parasites

An antigenic preparation of adult *Schistosoma mansoni*, *Fasciola gigantica*, and *Ascaris lumbricoides*, was prepared as described by Attallah et al.[30] The protein content of each antigenic preparation was determined and then aliquots were stored at -70°C until used.

Production of Anti-Toxoplasma IgG Antibodies

Specific IgG antibodies were produced in New Zealand white rabbits immunized subcutaneously in three different inoculation sites with crude Tachyzoites antigen or the purified 36-kDa antigen (see next section). In brief, equal volumes (500 μL) of the antigen (500 μg/mL) and complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA) were homogenized together using two Luer-lock syringes connected to a 3-way stainless steel valve. Each rabbit was immunized subcutaneously, three times, once with antigen in CFA (on day 0) and twice with antigen in IFA (on days 15 and 28), before being killed on day 32. Blood samples were collected from all rabbits at 0, 28, and 32 days of immunization. Sera collected from the rabbits before immunization were used as a negative control. The reactivity of the collected rabbit sera was tested against the crude Tachyzoites antigen, the purified 36-kDa antigen, and serum samples of *Toxoplasma* infected individuals using ELISA (see sections below). The specificity was tested against crude antigens of *S. mansoni, F. gigantica*, and *A. lumbricoides*, and sera of healthy individuals and individuals infected with other parasites using ELISA. Sera were separated and stored at -20°C until used.

SDS-PAGE and Gel Electroelution

Samples (an extract of *T. gondii* tachyzoites and selected sera of women with different TSP) were subjected to analytical SDS-PAGE, at 50 μg/lane, using vertical slabs of 12% or 16% polyacrylamide.[31] Molecular-weight standards (Sigma) were run in parallel. In preparative slab gel electrophoresis, the running condition was adapted to reduce smear of proteins and to enable a considerable long migration distance between bands in the 36-kDa region in crude Tachyzoites antigen or serum sample according to the prestained molecular weight marker. In each run, a lane from electrophoresed preparative gel was Coomassie blue or Silver stained and immunoblotted to identify the 36-kDa band. In the unstained preparative gel, the adjacent band was then cut and the 36-kDa antigen electroeluted from polyacrylamide gel at 200 volts for 3 hr in a dialysis bag (Sigma). After dialysis, the electroeluted antigen was concentrated using polyethylene glycol and 40% trichloroacetic acid (TCA), then centrifuged at 6,500 × g for 15 min. The precipitate was washed twice using diethyl ether to remove the excess of TCA. The excess diethyl ether was removed by gentle drying and the pellet was reconstituted in phosphate buffered saline (PBS, pH 7.2). The protein content of a sample of electroeluted antigen was determined before remainder was stored at -20°C.

Western Blot

Samples separated on SDS-PAGE (as above) were electrotransferred onto nitrocellulose (NC) membrane (0.45 μm pore size, Sigma) in a protein transfer unit.[32] The NC membrane was blocked using 5% (w/v) non-fat dry milk dissolved in 0.05 M Tris-buffered saline (TBS) containing 200 mM NaCl (pH 7.4), rinsed in TBS and incubated with anti-*Toxoplasma* IgG antibodies diluted in blocking buffer with constant shaking. The NC membrane was washed 3 times (30 min each) in TBS, followed by incubation for 2 h with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:350 in TBS. After washing 3 more times with TBS (15 min each), the NC membrane was soaked in substrate (premixed 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT) in 0.1M Tris buffer, pH 9.6; ABC Diagnostics, New Damietta, Egypt). The color reaction was observed within 10 min, and dipping the NC membrane in distilled water then stopped the substrate reaction.

Characteristics of the Purified Toxoplasma Antigen

Purity

The purity of the purified *Toxoplasma* antigen was assessed using analytical SDS-PAGE[31] and capillary zone electrophoresis (CZE). In each CZE run, 10 μL of the purified antigen (50 μg/mL in distilled water) were injected through a 65 cm fused silica capillary (75 μm inner diameter) coated with polyimide film at high voltage (30 kV) and low pressure (25 mbar) for 10 seconds before the sample was eluted with borate buffer (pH 8.3) at high voltage for 15 min using an autosampler (model 1-LIFT, Prince Technologies, Emmen, The Netherlands). Eluents were detected by their ultraviolet absorption at 200 nm using a variable wavelength ultraviolet-visible detector (Lambda 1010, Metrhom, Herisau, Switzerland), and the signals were analyzed using Dax software (version 5; Prince Technologies).

Chemical Type

To determine some of the purified *Toxoplasma* antigen's biochemical characteristics, samples of the *Toxoplasma* antigen were treated with protease and one of several other chemical reagents being tested in the ELISA, to see if these treatments affected the active epitope. The purified antigen, at 1 mg/mL, was incubated for 1 hr, either with 40% TCA (v/v) at 4°C or with 0.2 M NaOH or 0.2 M HCl (v/v) at room temperature. A periodate oxidation was carried out overnight with 20 mM sodium metaperiodate at RT and the reaction was then inhibited by adding an equal volume of 130 mM glycerol. In another series of experiments, a sample of the antigen

(at 200 μg/mL) was mixed with an equal volume of 20, 60, or 180 mM β-Mercaptoethanol. In the test with protease, purified antigen (1 mg/mL) was incubated at 37°C with α-Chymotrypsin (1 mg/mL; Sigma) for 15, 30, 45, or 60 min. Tachyzoites and bovine serum albumin were tested in parallel, as positive and negative controls, respectively.

Reactivity of the Developed Anti-36-KDa Toxoplasma Antibodies

The reactivity towards the 36-kDa *Toxoplasma* antigen of the antibodies raised in rabbits against the purified 36-kDa antigen and the levels of their reactivity with antigens from other parasites were explored using ELISA as follows. After optimization of reaction conditions, flat bottomed, polystyrene, microtiter plates (Costar, Corning Life Sciences, Acton, MA) were coated with the 36 kDa antigen, *T. gondii* Tachyzoites antigen, or crude antigens of *S. mansoni, F. gigantica* and *A. lumbricoides* (2.5-μg/mL carbonate buffer, pH, 9.6). After blocking, 50 μL/well of a 1:1,000 dilution, in PBS with 0.05% (v/v) Tween 20 (PBS-T20), of a serum collected from a rabbit immunized with the 36 kDa antigen were added to each well. Normal sera from non-immunized rabbits were used as negative controls. The plates were incubated at 37°C for 2 hr, washed, and then incubated at 37°C for 1 hr with anti-rabbit IgG alkaline phosphatase conjugate (The Binding Site, Birmingham, UK) diluted 1:1,000 in PBS-T20. After washing, the substrate (1 mg/mL p-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4) was added and the plates were incubated for 20 min at 37°C. Optical densities (OD) were read at 405 nm using a microplate autoreader (Σ960; Axiom, Burstadt, Germany). Sera of 4 normal rabbits were used to set the cutoff limit of ELISA. The cutoff OD for ELISA positivity was set as 0.221, the mean OD plus three SD for the sera from four normal rabbits.

ELISA for Direct Detection of Circulating Toxoplasma Antigen (TAG) in Serum Samples

After optimization of ELISA conditions, polystyrene microtiter plates were coated with human serum samples diluted 1:800 in 50 mM carbonate buffer, pH 9.6. The plates were incubated overnight at 4°C. After incubation, the plates were washed four times with 0.05% PBS-T and residual PBS-T was then removed by tapping gently onto absorbent paper towels. Then, the plates were incubated for 1 hr at room temperature with 200 μL/well of 0.5% (w/v) BSA in PBS. The plates were washed 5 times (5 min each) and used immediately. Specific anti-*Toxoplasma* antibody was diluted 1:100 in PBS-T and incubated at 37°C for 2 h. After washing, 50 μL/well of anti-rabbit IgG alkaline phosphatase conjugate (Sigma) was diluted (1:400) in

0.2% (w/v) BSA in PBS-T, it was added and incubated at 37°C for 1 h. Excess conjugate was removed by extensive washing and the amount of coupled conjugate was determined by incubation with 1 mg/mL p-nitrophenyl phosphate in substrate buffer for 30 min at 37°C. The reaction was stopped by addition of 25 μL/well of 3M NaOH and the absorbance was read at 405 nm. The cutoff level of TAg-ELISA, above or below which the tested sample is considered positive or negative, was calculated as the mean OD for ELISA (range, 0.135to 0.377) of a group of 24 serum samples from *Toxoplasma* non-infected women $\pm$ 3 standard deviations (i.e., $0.231 \pm (3 \times 0.075)) = 0.306$. The *Toxoplasma* non-infected control women were parasitologically diagnosed infected with other parasitic infections, including schistosomiasis mansoni (n = 6), fascioliasis (n = 4), ascariasis (n = 2), giardiasis (n = 2), entamoebiasis (n = 4) and with no parasitic infection (n = 6). The mean OD value of a group of 32 sera from clinically and serologically diagnosed *T. gondii*-infected women was 0.655 (range, 0.354 to 1.125).

Statistical Analyses

Data were expressed as mean $\pm$ SD and were analyzed by using the statistical analysis program package, Instate Software for Science, version 2.3 (Graphpad Software, Inc., San Diego, CA). P values < 0.05 were considered significant.

RESULTS

Identification of the Target Toxoplasma Antigen

The rabbit anti-*T. gondii* Tachyzoites IgG antibody reacted with several bands in *T. gondii* Tachyzoites antigen of varying molecular weights on the Western blot. However, a band representing one antigen of 36 kDa, showed particularly high immunoreactivity (Fig. 1). The 36-kDa antigen was purified from Tachyzoites antigen using electroelution, allowing monospecific anti-sera to be developed in rabbits immunized with the purified antigen. In Western blot with Tachyzoites antigen, the anti-36 kDa sera only appeared to react with an antigen of 36 kDa (Fig. 1). In ELISA, these anti-36 kDa antibodies showed high reactivity towards Tachyzoites antigen and the purified 36 kDa antigen, but gave titers below the threshold for positivity when tested against the antigens from *Schistosoma, Fasciola*, and *Ascaris*. The normal rabbit sera showed no reactivity towards the tested antigens. The purified 36 kDa antigen was analyzed by analytical SDS-PAGE and stained with silver stain. A single polypeptide band was silver stained at 36 kDa molecular weight and its reactivity was confirmed using Western blot

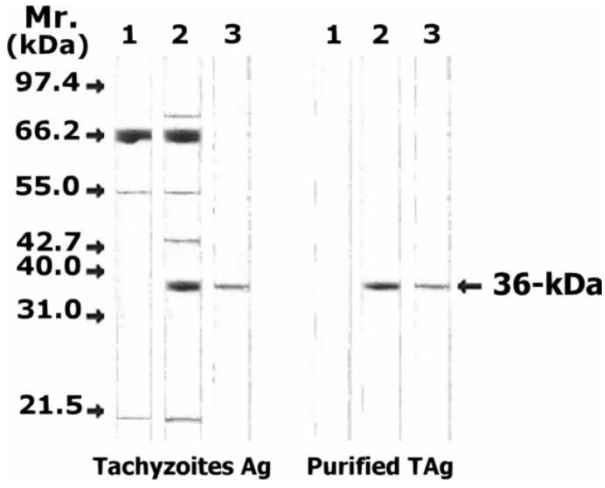

Figure 1. Western blot analysis of *T. gondii* tachyzoites antigen (Tachyzoites Ag) and the purified 36-kDa *Toxoplasma* antigen (Purified TAg) by using specific antibodies. A highly reactive band was identified at 36 kDa using rabbit anti-*T. gondii* tachyzoites IgG antibodies and rabbit anti-36 kDa antigen IgG antibodies. Lane 1: immunostained with normal rabbit serum; lane 2: immunostained with anti-Tachyzoites antibodies and lane 3: immunostained with anti-36 kDa antigen antibody. Molecular weight markers (not shown but indicated by arrows) include phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa), aldolase (40 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa).

(Fig. 2a). The purity of the eluted 36 kDa antigen was confirmed using CZE. A single CZE peak was shown at 10.9 min (Fig. 2b). Selected sera of women with confirmed acute *T. gondii* infection ($n = 5$) and of non infected women ($n = 5$) were tested against the monospecific antisera raised against the 36 kDa antigen using Western blot. An intense and sharp reaction was shown at 36 kDa molecular weight band in all serum samples of acutely infected women, but no reaction was shown in sera of non-infected women (Fig. 3a). The 36 kDa antigen was purified from serum by electroelution from preparative polyacrylamide gels. The purity of eluted serum antigen was assessed using SDS-PAGE and its reactivity was confirmed using Western blot (Fig. 3b).

Partial Characterization of the 36 kDa Toxoplasma Antigen

Temperatures up to 56°C didn't alter the recognition of the reactive epitope of the purified 36 kDa *Toxoplasma* antigen from tachyzoites antigen by specific

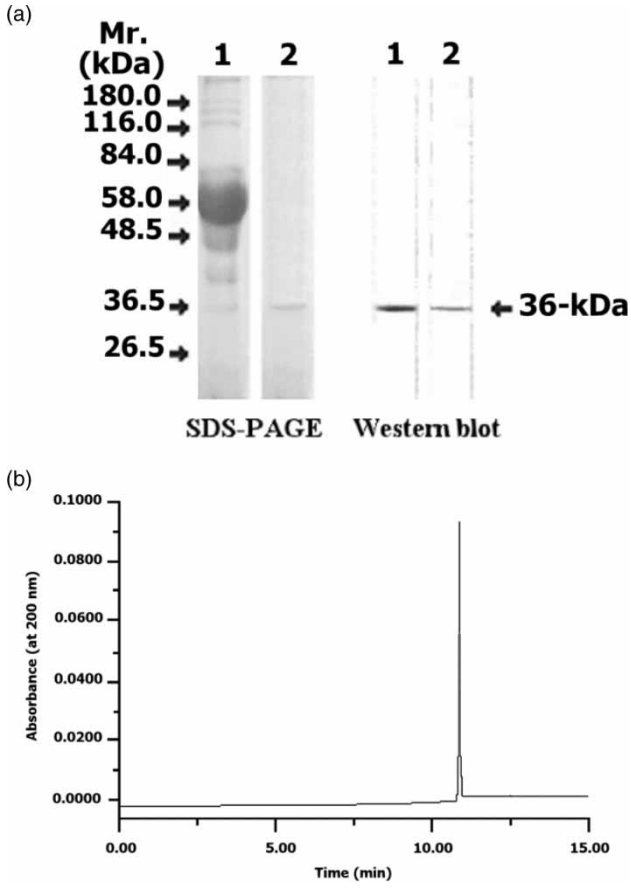

Figure 2. Purification of the 36 kDa *Toxoplasma* antigen from *T. gondii* tachyzoites antigen. (a) Silver stained SDS-PAGE and western blot of the purified 36 kDa antigen. Lane 1: crude *T. gondii* tachyzoites antigen, lane 2: Purified 36 kDa fraction. Molecular weight markers (Mr.) include: α-2-macroglobulin (180 kDa), β-galactosidase (116 kDa), fructose-6-phosphate kinase (84 kDa), pyruvate kinase (58 kDa), Fumarase (48.5 kDa), lactic dehydrogenase (36.5 kDa), triose phosphate isomerase (26.5 kDa). (b) CZE electropherogram showing a single peak at 10.9 min confirming the purity of the 36 kDa fraction from Tachyzoites antigen. The serum fraction showed also a single peak at 10.9 min.

anti-*Toxoplasma* antibody by using ELISA. Higher temperatures resulted in loss of antibody reactivity, indicating a conformational nature of the target epitope. The reactivity of specific anti-*Toxoplasma* antibody was also lost towards the purified *Toxoplasma* antigen treated with acid or alkali. However, antibody reactivity towards purified *Toxoplasma* antigens was maintained after metaperiodate oxidization and reduction with

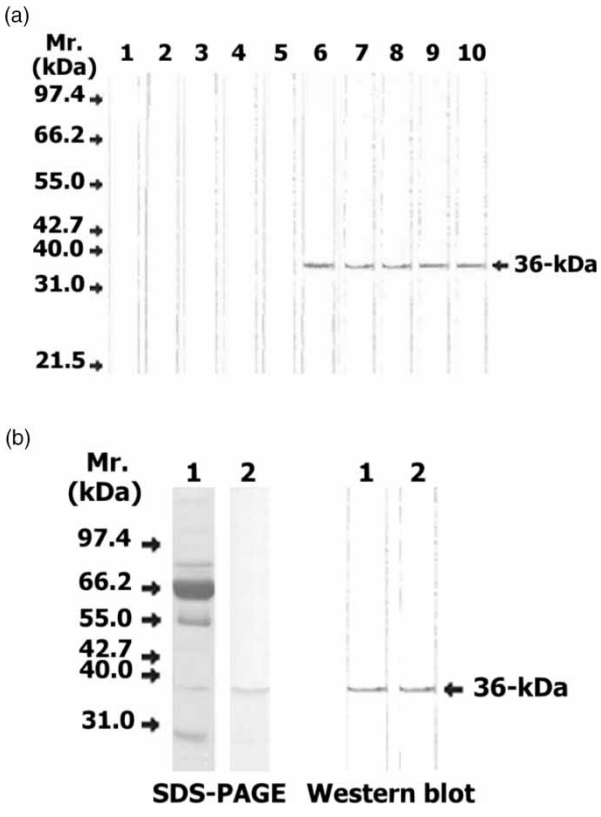

Figure 3. Identification and isolation of the target 36 kDa *Toxoplasma* circulating antigen from serum. (a) Western blots of selected serum samples from non-infected and acutely *T. gondii* infected women. Lanes 1–5: Sera of 5 non-infected women. Lanes 6–10: Sera of 5 acutely *T. gondii* infected women. (b) Silver stained SDS-PAGE and Western blot of the 36 kDa antigen purified from serum. Lane 1: Serum of *T. gondii* infected woman, and lane 2: Purified 36-kDa fraction from human serum. Molecular weight markers (Mr.) include: phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55 kDa), ovalbumin (42.7 kDa), aldolase (40 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa).

β-mercaptoethanol. The TCA precipitate fraction gave the same reactivity that has been shown for the purified *Toxoplasma* antigen, but the TCA supernatant fraction showed no reactivity. The reactivity was decreased by increasing the incubation time with α-chymotrypsin enzyme and was completely lost at 60 min incubation. The 36 kDa antigen, purified from serum, showed similar biochemical properties to the 36 kDa antigen produced by tachyzoites using the same techniques.

Detection of 36 kDa Toxoplasma Circulating Antigen in Sera of Infected and Non-Infected Individuals Using ELISA

A simple ELISA format was adapted for the detection of the circulating *Toxoplasma* antigen (TAg-ELISA) in sera of clinically and serologically diagnosed 222 women; see Table 1. The circulating *Toxoplasma* antigen was detected in 88% of women with acute infection and in 66% of chronically infected women. Using the more sensitive Western blot, the 36 kDa *Toxoplasma* circulating antigen was identified in all sera of 17 women with acute infection and showing negative TAg-ELISA results and in 10 sera of 15 women with chronic infection and showing negative TAg-ELISA results; see Fig. 4. The specificity of the TAg-ELISA test was 91% among sera of 32 *Toxoplasma* non-infected women and parasitologically diagnosed infected with other parasitic infections, including schistosomiasis mansoni ($n = 8$), giardiasis ($n = 5$), entamoebiasis ($n = 10$) and with no parasitic infection ($n = 9$). The samples showing false positive results (9%) using TAg-ELISA were of two women with entamoebiasis and one woman with no parasitic infection. Using Western blot, the 36 kDa *Toxoplasma* circulating antigen was not identified in all sera of 3 non-infected women showing positive TAg-ELISA results. No significant correlation was shown between the levels (expressed in optical density units) of IgM (or IgG) antibodies and that of the 36 kDa *Toxoplasma* circulating antigen (Spearman $r = 0.11$, $p > 0.05$).

DISCUSSION

Acquisition of *Toxoplasma* infection during pregnancy requires intervention and treatment. To provide proper clinical care for these pregnant women, it

Table 1. Detection of circulating *Toxoplasma* antigen (TAg) in 222 sera of *Toxoplasma gondii* infected and non-infected women using TAg-ELISA

		TAg-ELISA		
Status of *T. gondii* infection[a]	No.	Positive	Negative	% Positive
***Toxoplasma* infected individuals**				
Acute infection	146	129	17	88%
Chronic infection	44	29	15	66%
Non infected individuals				
No parasitic infection	10	1	9	10%
Other parasitic infection	22	2	20	9%
Total	32	3	29	9%[b]

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

[b]Revealed 91% specificity of TAg-ELISA.

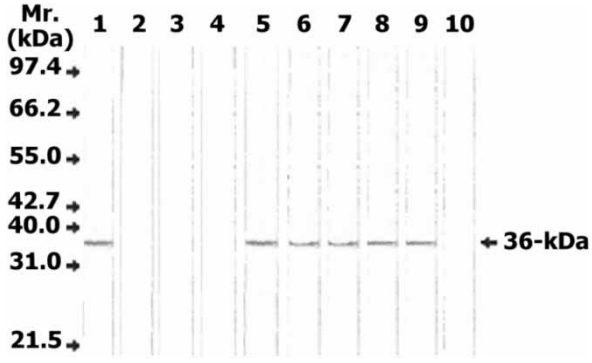

Figure 4. Detection of the 36 kDa *Toxoplasma* antigen in serum samples showing false positive or negative TAg-ELISA results using the more sensitive western blot. Lane 1: crude *T. gondii* Tachyzoites antigen. Lanes 2–4: Serum samples of the 3 non-infected women showing positive results using TAg-ELISA. Lanes 5–7: Selected serum samples of woman with acute *Toxoplasma* infection showing negative TAg-ELISA results. Lanes 8–10: Selected serum samples of women with chronic infection showing negative TAg-ELISA results. The target 36-kDa antigen was identified in all sera of acutely infected women and in most of chronically infected women but not identified in all sera of non-infected women. Molecular weight markers (Mr.) were not shown but indicated by arrows.

is important to have an accurate method for diagnosis of active *Toxoplasma* infection.[33] An attractive alternative to traditional serology is the direct detection of cellular components and metabolites of the parasite, which are present in the blood stream and, consequently, be shedding into the urine during the acute phase of infection and are called circulating antigens.[17,34,35] The identification and detection of circulating antigens may prove very useful in the diagnosis of the acute phase of infection by *T. gondii*, as well as during reinfections, and could pave the way for early treatment and, consequently, effective control of the disease.[36] In the present study, a specific rabbit anti-serum and Western blot analyses were used to demonstrate the presence of a highly reactive antigen of 36 kDa, not only in an extract of *T. gondii* Tachyzoites, but also in sera of women with confirmed laboratory and clinical signs of recent Toxoplasmosis. The cluster of *Toxoplasma* antigens in the range of 28–36 kDa gained a specific interest.[24,25,37] These antigenic markers of *T. gondii* were recognized by human IgM antibodies in serum samples from patients with acute Toxoplasmosis.[7,38] Our 36 kDa protein from *T. gondii*, approximately, has a closely related molecular weight to the 35 kDa *Toxoplasma* antigen[7,23] and a novel *T. gondii* rhoptry protein (named ROP9) recently defined as a 36 kDa protein (p36) present in the soluble fraction of tachyzoite lysate, but not in bradyzoites.[39] ROP9 is soluble within the parasite and in infected

cells, targets to the parasitophorous vacuole membrane, and may be involved in the early steps during host-cell invasion. It is of interest that the identification of a 35 or 36 kDa *Toxoplasma* antigen in serum samples from women infected with *T. gondii* was not reported before, as far as we know. The purified 36 kDa serum antigen showed similar biochemical properties to the 36 kDa antigen produced by tachyzoites. However, further study, including amino acid analyses and sequence, will be performed to confirm the identity. The diagnostic potential of *Toxoplasma* circulating antigens were explored in few reports. However, the methods used were rather elaborate and expensive, which may not be suitable for routine examination.[35] In the present work, an ELISA format was developed for the direct detection of the 36 kDa target *Toxoplasma* antigen (TAg-ELISA) in selected serum samples of serologically diagnosed 222 pregnant women. The TAg-ELISA detected *Toxoplasma* antigen in 88% of the sera of women with acute *Toxoplasma* infection, and in a high percentage (66%) of women with chronic infection. The reported *Toxoplasma* antigen detection rate (88%) in samples containing IgM antibodies was much higher than the previously reported rates (about 60%) in acutely infected cases,[26,27] and it was similar to the one (90%) recently reported by Susanto and Muljono.[28] All sera of women with acute *Toxoplasma* infection and showing negative TAg-ELISA results were positive using the highly sensitive Western blot. The low levels of target *Toxoplasma* circulating antigen may explain the false negative results of TAg-ELISA in sera of these women, or these sera may represent a case of chronic *Toxoplasma* infection with IgM persistence for a long time.[9,10,40] Surprisingly, the *Toxoplasma* antigen detection rate in chronically *Toxoplasma* infected women was much higher than those rates reported by other investigators in samples containing IgG only.[27,28] The 36 kDa *Toxoplasma* antigen was also identified using the highly sensitive Western blot in most sera of these women showing negative TAg-ELISA results. The presence of high titers of specific IgG antibodies in the sera of infected individuals does not indicate a complete elimination of the pathogen; such high titers of antibodies may not neutralize all of the circulating antigens and, also, the specific IgM antibodies' response will not be stimulated for new infections.[41] The developed ELISA showed a high degree of specificity (91%) using sera of women negative for both *Toxoplasma*-specific IgM and IgG antibodies and who were parasitologically diagnosed to be infected with, or without, other parasites. However, a large number of control individuals is required to draw final conclusions. In conclusion, we have identified a 36 kDa Toxoplasma circulating antigen in sera of infected individuals. The detection of target *Toxoplasma* antigen in sera of infected women by ELISA appears to be a promising alternative approach for laboratory diagnosis of active *T. gondii* infection. Further studies regarding the clinical utility of the new *Toxoplasma* circulating antigen detection immunoassay and the assessment of the target antigen after specific treatment of *T. gondii* infected individuals will be performed.

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