

Home-Made Western Blot Assay Versus Commercial Fast Dot-ELISA Technique in the Diagnosis of Human Toxoplasmosis

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Abstract: Toxoplasmosis can be diagnosed indirectly with serological methods with detection of specific *Toxoplasma* IgG, IgM and IgA antibodies, yet those techniques are not reliable enough to detect specific anti-*T. gondii* antibodies during the active phase of the parasitic infection. In the current study, we have purified a specific *T. gondii* antigen (s) as to detect its efficacy in diagnosis of the disease at the hardly detectable phase. ELISA and immunoblot assays in parallel with a developed home-made dot ELISA were used to achieve our goal. A total of 153 human sera were collected from patients and assigned into three groups: A first group of 88 sera pooled from *T. gondii*-infected patients, a second group of 35 samples collected from patients infected with other parasites and, ultimately, a third group of 30 sera pooled from healthy individuals (control). Our results showed a comparable significance in the sensitivity, specificity and efficacy obtained by using the commercial kit and home-made dot ELISA. Hence our study suggests that utilizing purified *Toxoplasma* antigenic fraction might be a valuable protein in diagnosis of toxoplasmosis indirectly through detecting specific anti *Toxoplasma* IgG and IgM antibodies.

Key words: Toxoplasmosis • *Toxoplasma gondii* • Diagnosis • ELISA • Dot-ELISA

INTRODUCTION

Toxoplasmosis is caused by an obligate intracellular protozoan parasite *Toxoplasma gondii* (*T. gondii*) and one of the most common parasitic infections of humans and other warm-blooded animals [1]. The infection is generally asymptomatic in the immunocompetent individual but when acquired congenitally or as a consequence of reactivation in immunocompromised patients, toxoplasmosis can be life-threatening [2]. In the latter patients, the parasite causes the commonest opportunistic infection with clinic neurological complications between 39-70% [3, 4].

In pregnant women, the primary infection of *T. gondii* may cause abortion, neonatal malformation, neonatal death, or severe congenital deficiency, such as mental retardation, retinochoroiditis and blindness [1, 5, 6]. The clinical severity of this parasitic infection during pregnancy is gestational time-dependent. Therefore, it is important to be diagnosed early and to distinguish between acute and chronic stages of infection for treatment and limitation of the effects [7].

Toxoplasmosis can be diagnosed indirectly with serological methods with detection of specific *Toxoplasma* IgG, IgM and IgA antibodies [8] and directly by polymerase chain reaction (PCR), hybridization, isolation and histology. The indirect serological methods are widely used in immunocompetent patients, whereas the definitive diagnosis in immunocompromised people is mostly undertaken by direct detection of the parasite [9].

Serological methods are not reliable enough to detect specific anti-*T. gondii* antibodies during the active phase of the parasitic infection for many reasons [10]. First, antibodies are produced just several weeks before parasitemia. In a congenital toxoplasmosis, for example, the pregnant mother's test might be negative during the active phase of *T. gondii* infection and as a consequence the fetus would be subjected to death. Likely, in immunocompromised patients, the test might be negative because of the very low titer of specific antibodies anti-*T. gondii*. Even if produced early like IgM antibodies anti-*T. gondii*, it could not be an indicator of the early/late phase of the diseases as its titer persists for long periods of time that could extend from months to years [4, 11].

Second, false positive reactions might occur with antinuclear antibodies, rheumatoid factors, or naturally occurring human antibodies. Ultimately, the false negative reactivity might also rise from competitive inhibition by high levels of specific IgG antibodies [12]. Consequently, rapid diagnosis of *T. gondii* infection can be conducted not only by measuring antibody levels but by detecting the parasite and/or its components as well. This might help treating the disease at early stage and reduce the clinical severity and damage of the acute toxoplasmosis [13]. There are many serologic kits commercially available for diagnosis of *T. gondii* infection based primarily on the use of whole extracts of tachyzoites. However, these extracts contained undesirable materials such as large amount of proteins and other macromolecules; these materials result in pseudo positive results. Thus, it is obviously necessary to produce large quantity of specific purified antigens in order to diagnose the human toxoplasmosis with high sensitivity [6].

The aim of this study was to develop a specific *T. gondii* antigen (s) as to diagnose the disease at the early active phase. To approach this goal, *T. gondii* antigen was purified and its efficacy in the detection of immunoglobulins G and Immunoglobulins M antibodies by indirect ELISA, home-made Dot ELISA and immunoblot assays was determined.

MATERIALS AND METHODS

Serum Sample Collection: On the basis of clinical history and positive serological tests or microscopic examination, a total of 153 human sera were collected from patients admitted to the following Departments of Kasr Alini University Hospital: The obstetrics and gynaecology, Infectious Diseases, Ophthalmology and dialysis. Those samples were assigned into three groups: A group of 88 sera pooled from *T. gondii*-infected patients, another group of 35 samples collected from patients infected with other parasites and, ultimately, a third group of 30 sera pooled from healthy individuals (control).

Preparation of Toxoplasma Antigen: *T. gondii* RH tachyzoites; a gift from the Faculty of Veterinary Medicine, Cairo University; were grown and maintained in three Swiss Albino mice ascites by intraperitoneal inoculation. Every 4 days after infection, the peritoneal fluids from infected mice were collected in saline, pooled and centrifuged at 1000 g for 10 min. The parasite pellets were washed twice, counted and suspended in saline and inoculated in 0.2ml into the peritoneal cavity of 3 other

mice to maintain the tachyzoites [14]. Tachyzoites were collected from the peritoneal exudates in sterile PBS, pH 7.2. The organisms were centrifuged at 2000g for 20min, washed three times in PBS and disrupted in the cold by sonication. Lysed cells were centrifuged at 12000g for 1hr at 4°C. The supernatant was collected and used as the soluble *T. gondii* antigen [15]. *Toxoplasma* antigens were identified by Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. 20ug/well of protein fraction from purified *Toxoplasma* tachyzoites antigen (PTT) in 20 µl of sample buffer consisting of 0.5 M Tris-HCl pH 6.8 and supplemented with 0.8 ml glycerol, 1.6 ml 10% SDS, 0.4 ml B- mercaptoethanol and 0.4 ml 0.05% bromophenol blue, was electrophoresed on 12% SDS-PAGE. The antigen bands and molecular weight markers were visualized in polyacrylamide gel by staining the gel with Coomassie brilliant blue R-250 (Sigma).

Indirect ELISA: Indirect ELISA was performed to measure the anti-*T. gondii* IgG, IgG4 and IgM level according to the standard protocol. All reaction steps, except coating and washing, were performed at 100µl per well of the PTT antigen in a concentration of 5µg/ml at 4°C overnight. The plates were washed five times with PBS containing 0.05% Tween20 and blocked for 3–4 h at 37°C with 100µl per well of 0.5% BSA in PBS. After washing, 100µl of each serum sample, diluted at 1:50 was added to the well in triplicates for 1h. The plates were washed 5 times with the washing buffer. 100µl of goat anti-human (IgG or IgG4 or IgM) conjugated to horse radish peroxidase (HRP, secondary antibody), previously diluted at 1:2000 in washing buffer, was added to each well and incubated for 1hr at 37°C. After 5 times washing, a color reaction was observed by incorporating 0-phenylenediamine dihydrochloride (Sigma) into the reaction, which was stopped by adding 50µl of 2M H₂SO₄. The optical density was measured at 492 nm (OD₄₉₂) in a microtitre plate ELISA reader (Bio-Rad microplate reader, Richmond, CA).

Home-Made dot ELISA: Five µl of the PTT antigen was dotted on nitrocellulose membrane discs and allowed to air dry thoroughly. The Dot-ELISA test was done by the method described earlier by Yamamoto *et al.* [17]. The discs placed into flat bottom micrometer plate well. Non-specific binding sites were blocked by adding 100µl of PBS containing 0.5% tween20 and 1% bovine serum albumin to each well then incubated 1h in 37°C. 100µl of the serum samples were diluted with PBS-Tween in double dilution starting from 1:100 and were added to the discs

and the plate was incubated at room temperature for 1h. The discs were washed again with PBS-Tween and 100µl of the 1:1000 goat anti-human (IgG or IgM) conjugated to horse radish peroxidase (Sigma) was added to each well and the plate incubated for 2h. After washing, 100µl of 0.5mg/ml DAB (Diamino Benzidine tetrahydrochloride), 0.03% H₂O₂ in PBS were added and incubated for 30min. Serum dilutions that gave visible brown spots on discs at titers ≥ 400 were considered positive. For calculation of sensitivity, specificity, rate of false positive, rate of false negative, positive predictive and negative predictive values standard formulas were used.

Commercial *T. gondii* IgG/IgM Kits: Commercial *T. gondii* IgM/IgG Kits (Haitai Biological Pharmaceuticals Co., Ltd, China) was used to verify the data of the home-made dot ELISA by detecting human IgG and IgM in all samples by the Western blot and the soluble extract of *T. gondii*. The procedure of the commercial IgG/IgM kit was performed according to the manufacturer's instructions. Briefly, the parasite-derived soluble proteins were separated on a 12% SDS-PAGE gel and transferred onto nitrocellulose membranes (Bio-Rad, CA, USA). The membrane was cut into strips and incubated with the positive and negative sera (at 1:50 dilution) identified in the ELISA assays. Meanwhile, a serum of an individual previously confirmed with *T. gondii* infection was used as a positive control. The membrane strips were further incubated with an alkaline phosphatase-conjugated goat anti-human IgG/IgM antibody (1:5000 dilutions) after washing in TBST buffer (10mM Tris, 150mM NaCl, pH 8.0 and 0.05% Tween20). Eventually, the strips were incubated with BCIP/NBT substrate solution to visualize the protein bands that were recognized by the specific antibodies.

RESULTS

The purified *Toxoplasma* tachyzoites antigen (PTT) was analyzed and demonstrated with the SDS-PAGE analysis and Coomassie brilliant blue staining (Fig. 1). PTT showed several polypeptide bands ranged from 110 to 12 KDa. Five major bands were detected at 95, 49, 38, 28 & 12 KDa. On the other hand, the analysis of PTT by immune blot assay against *T. gondii*-infected patients sera recorded high frequency of reactivity with the following bands: 95KDa (100%), 49KDa (87.8%), 27KDa (92.6%) and 12KDa (85.3%). The least reactivity was due to 38KDa (17%) as depicted in Table (1).

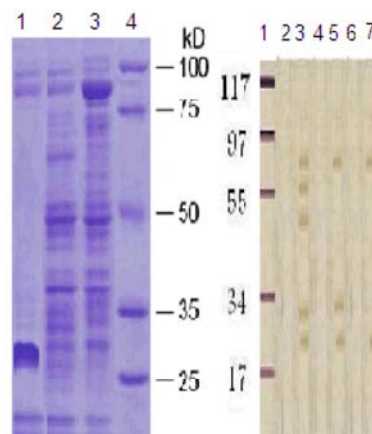


Fig. 1: SDS-PAGE analysis and immune blot assay of PTT antigen

A) 1. Molecular weight St., 2. Crude toxoplasma fluid; 3. partially purified toxoplasma fluid antigen, 4. purified toxoplasma fluid antigen. B) 1. Standard, 2. Negative sera with Crude Ag, 3. Positive sera with Crude Ag, 4. Negative sera with partially purified Host free Ag, 5. Positive sera with partially purified Ag 6-Negative sera with purified Ag, 7-Positive sera with purified Ag.

Indirect ELISA: In order to measure the incidence of positivity for the anti *T. gondii* IgG, IgG4 and IgM level in a total of 153 human sera, it was necessary at first to determine the cut off point for positivity or the line of demarcation between positive and negative data. The cut off point for positivity was measured as mean OD reading of negative controls + (2) standard deviation (SD). The tested samples showing OD values > cut off value were considered positive for *T. gondii*.

Table (2 & 3) shows the results of the anti-*T. gondii* IgG, IgG4 and IgM level in the tested sera of all studied groups. The cut off values for positivity were 0.368 ± 0.202 , 0.297 ± 0.192 and 0.278 ± 0.132 for IgG, IgG4 and IgM; respectively. Out of total 88 *Toxoplasma*-infected sera, 73 were positive to anti-IgG, 75 were in favor of anti-IgG4 and 68 were positive to anti-IgM. The average sensitivity of PTT antigens to IgG, IgG4 and IgM was 82.9%, 85.2% and 77.3% respectively. On the other hand, the false positive data were counted among the total 65 negative samples recorded in the study, including 15, 8 and 13 false positive results for the anti-*T. gondii* IgG, IgG4 and IgM, respectively. The average specificity of PTT antigens to IgG, IgG4 and IgM was 76.9%, 87.7% and 80% and diagnostic accuracy of PTT antigen was 85.2%, 87.7% and 85.7% respectively (Table. 2).

Table 1: Reactivity of the five major bands by immune blot assay against *T. gondii*-infected sera

MW	95 KDa	49 KDa	38 KDa	27 KDa	12 KDa
Sensitivity	100	87.8	17	92.6	85.3
Specificity	88	84	72	68	72
PPV	93.1	87.8	50	82	83
NPV	100	80.7	34.6	85.7	83.5

Table 2: Evaluation of sensitivity to IgG, IgG4 and IgM in the sera of all cases studied

Groups	Positive cases (Sensitivity%)			Negative cases (Sensitivity%)		
	IgG	IgG4	IgM	IgG	IgG4	IgM
Healthy control (n= 30)	--	--	-	30	30	30
Toxoplasma (n= 88)	73	75	68	15	13	20
Entameaba histolytica (n= 17)	10	5	8	78	83	80
Giardia lamblia (n= 18)	5	3	5	83	85	83

Table 3: Detection of specificity and positivity of purified *T. gondii* antigen by Indirect ELISA

Evaluation	IgG	IgG4	IgM
Sensitivity	82.9%	85.2%	77.3%
Specificity	76.9%	87.7%	80%
Diagnostic Efficacy	80.5%	85.7	78.5%

Table 4: Positive and negative cases demonstrated by the commercial kit versus the home-made kit

Group	Commercial kit				Home-made kit			
	IgG		IgM		IgG		IgM	
	+ve	-ve	+ve	-ve	+ve	-ve	+ve	-ve
Healthy control (n= 30)	-	30	-	30	-	30	-	30
Toxoplasma (n= 88)	84	4	79	9	76	12	74	14
Entameaba histolytica (n= 17)	3	14	4	13	3	14	6	11
Giardia lamblia (n= 18)	1	17	2	16	2	15	4	14

Commercial Versus Home-made Kits (Tables 4 & 5; Fig. 2): Detection of the anti *T. gondii* IgG and IgM level among different studied groups in the serum samples by the commercial kit had been depicted in Tables 4 and Fig. 2. Highly positive 84 sera of the total 88 *Toxoplasma* infected cases demonstrated 95.5% sensitivity to anti-IgG and only 79 recorded 89.9% sensitivity to anti-IgM.

The specificity of the commercial kit was determined as the sum of results recorded from testing the negative control group (n= 30) and the group of samples infected with some other different parasites (2nd group, n= 35). Therefore, the specificity and diagnostic accuracy of the assay to IgG and IgM were 93.8% and 94.5% in favor of the first and 87.7% and 88.5% in favor of the latter (Table 5).

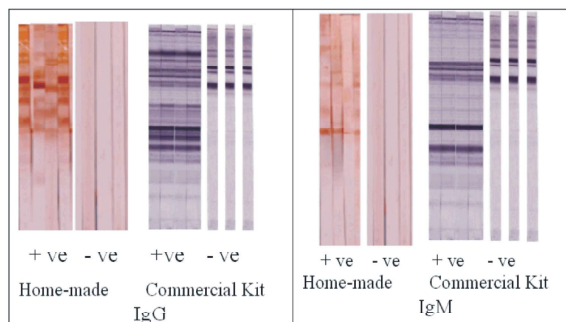


Fig. 2: Responses of IgG and IgM detection by both home-made kit (left) and Commercial Kit (right)

Table 5: Evaluation of the sensitivity, specificity and diagnostic efficacy to IgG and IgM in the studied cases

Evaluation	Commercial kit		Home-made kit	
	IgG	IgM	IgG	IgM
Sensitivity	95.5%	89.9%	86.4%	84.1%
Specificity	93.8%	87.7%	92.3%	84.6%
Diagnostic efficacy	94.5%	88.5%	90.8%	84.9%

The diagnostic performance of the home-made kit in detecting IgG and IgM responses to *T. gondii* PTT was compared to that detected for the commercial kit (Table 4 & Fig. 2). Seventy six of the 88 *Toxoplasma* infected cases with high positive IgG titer showed 86.4% sensitivity. All samples except five of the 2nd group had shown negative results to IgG. Therefore, the specificity and diagnostic accuracy of the assay were 92.3% and 90.8%; respectively. As for IgM, 74 of the 88 *Toxoplasma* infected cases with high positive to IgM titer reflected 84.1% sensitivity. On the other hand, all samples excluding 10 of the 2nd group had shown negative results to IgM; the specificity and diagnostic accuracy of the assay were 84.6% and 84.9% respectively (Table 5).

DISCUSSION

In the present study characterization of the purified toxoplasma tachyzoites (PTT) antigen using SDS-PAGE, revealed many bands ranged from 110 to 12 KDa with 5 foremost proteins of 95, 49, 38, 27 & 12 KDa MW. The previous studies showed several bands with variety of molecular weights, yet have different range than ours, from 17 and 105 [19] and 6 to 116 KDa [20]. On contrary to our findings [21], the main antigens detected by anti-*T. gondii* antibodies in newborn infants varied from 21-116 KDa and more prevalent antigens varied from 45-66 KDa and from 97-116. Data obtained from experimental *T. gondii*-infected mice revealed two common bands with

molecular weights of 97& 32 KDa at the 4th day post infection [22]. In the current research study, the western blot analysis applied to *T. gondii*-infected sera against PTT antigen using anti-human IgG revealed positive reaction at 95, 49, 28 and 12KDa bands with sensitivity of 100%, 87%, 92% and 85.3% and specificity of 88%, 84%, 68% and 72%, respectively. Our results along with others [22] indicate that the antigens of *T. gondii* that generate the specific immune antibody response might be in between 116 and 6. However, precisely, it was demonstrated that the the region of 27-29 KDa are commonly found in *Toxoplasma* infection [23, 24] and that antigen components of 90, 41 and 26 KDa reacted to all sera containing IgG [14]. In our study, the MW of separated proteins may differ from those detected by other studies because of many reasons such the Western blotting methodology that varies from one lab to another, the quality of the markers used, the condition of electrophoresis and percentage of polyacrylamide in SDS-PAGE gel [25] as well as the country origin of antigenic constitution of *T.gondii* isolates. Furthermore, the different strains might affect the outcome, for example the *T. gondii* RH strain produced dark bands of molecular weight 30 and 32 KDa in the blotting whereas the band detected from TRH strain of 80 KDa MW was darker [19]. Those differences could likely result in different antibody profiles [21], as well.

Likely to data reported by Younis *et al.* [27] and within the range of data published by others [28;, 29], our data pooled from indirect ELISA using the PTT antigen revealed 93% sensitivity for IgG. While the specificity recorded in our study was apparently less than theirs (56% vs 85%, 90% and 92% detected by each respectively) and in agreement with the low specificity (48.8%) obtained by PTT in diagnosis of *Toxoplasmi* encephalitis in a previous study [30]. The differences between the results of the present work and the others may be due to the difference in the method of antigen preparation, the strain of *T. gondii* and the uneven number of cases and controls (Group I & Group II).

Considering to the diagnosis of active toxoplasmosis in the present study by ELISA technique, the serological diagnosis of acute toxoplasmosis has traditionally been made by detection of specific IgM antibodies and the currently available commercial tests to detect different anti- *Toxoplasma* antibodies relay mostly on preparation of crude parasite antigen. However the use of the whole tachyzoites antigen can result in false positive reactions [31]. In this study the positive reactions obtained by

prepared PTT antigen with anti-*T. gondii* IgG, IgG4 and IgM level in a total of 153 human sera recorded average sensitivity of 82.9%, 85.2% and 77.3% respectively. The sensitivity percentage we detected are in agreement with the results of others [33, 34], however, the average specificity of PTT antigens to IgG, IgG4 and IgM was 76.9%, 87.7% and 80% and diagnostic accuracy of PTT antigen was 85.2%, 87.7% and 85.7% respectively. The specificity percentage is different from that (100%) obtained by Juma & Salman [33].

A comparison between the data obtained by the home-made kit and commercial kit had demonstrated that specificity and diagnostic accuracy of home-made assay to Ig G1 was 86.4%, 92.3% and 90.8%; respectively, whereas their counterparts of the commercial kit were 95.5%, 93.8% and 94.5%. On the other hand, the average sensitivity, specificity and diagnostic accuracy obtained by home-made kit in response to IgM was 84.1%, 84.6% and 84.9%, respectively, compared to 89.9% 87.7%% and 88.5% gained by applying the commercial kit. In some study [34], a sensitivity of 73.5% and specificity of 97.4% in diagnosis of congenital toxoplasmosis by IgG WB and another one [21] found that IgG immune-blotting gave a sensitivity and specificity of 88% and 89.2% respectively. Moreover, immunoblotting appeared to be an excellent alternative first line in seroconversion confirmation test in pregnant women with sensitivity of 92.3% and it can strongly detect IgG toxoplasmosis bands [35].

In conclusion, the ability of hHome-made immunoblotting assay to provide a sensitive and specific antibody reaction to *Toxoplasma* antigens during the course of infection might be a valuable addition to serological methods. Also, purified *Toxoplasma* antigenic fraction (PTT) considered an important protein in diagnosis of toxoplasmosis with high sensitivity and specificity, indirectly by detection of specific anti *Toxoplasma* IgG and IgM antibodies.

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