Detection of *Toxoplasma gondii* IgG antibodies in Thai patients by enzyme-linked immunosorbent and immunoblot assays

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Abstract

An in-house ELISA was developed for detection of IgG *Toxoplasma* antibody using either pellets and/or supernatant from sonicated *Toxoplasma* tachyzoites as crude antigen. This method was compared with with a commercial p-30 ELISA which uses the membtane protein p-30 protein as the predominant antigen. A total of 259 sera from 3 groups of patients were tested. The in-house ELISA using either parasite pellets or supernatant antigen showed equal sensitivity (87.0%) in detection of IgG antibodies to *Toxoplasma*. The specificities of pellet and supernatant-based assays were 97.9% and 95.3% respectively. By Kappa analysis, highly significant correlation coefficient was obtained between the in-house ELISA and the commercial test kit (Kappa correlation between the in-house ELISA using pellet as antigen and Platelia = 0.869, $p < 0.001$; that using the supernatant as antigen and Platelia = 0.822, $p < 0.001$). There was a good concordance between in-house ELISA and p-30 ELISA results. In addition, the usefulness of the immunoblot assay for the detection of IgG antibodies to *Toxoplasma* was evaluated. Both antigens show generally similar polypeptide bands. The protein component with a molecular weight of 31 kDa reacted strongly with IgG antibodies in the majority of *Toxoplasma* seropositive sera (75.3%). It may represent a specific marker for diagnosis of *Toxoplasma* infection in Thai patients.

Key words: *Toxoplasma gondii*; immunoblot; ELISA; IgG anti-*Toxoplasma* antibodies

Introduction

Toxoplasma gondii infection is often asymptomatic in healthy individuals but the disease may be severe or fatal in the developing foetus and immunocompromised individuals (Tanphaichitra, 1987; Johnson & Holliman, 1995). As the organism is difficult to isolate in toxoplasmosis patients, the primary diagnosis is based on demonstration of specific antibodies by serological tests (Hafid *et al.*, 1995; Fuentes *et al.*, 1996). Antigens prepared from sonicated *Toxoplasma* tachyzoites have been used for the development of serological diagnostic assays and for the study of host immune response to the parasite (Hughes *et al.*, 1982; Gross *et al.*, 1992). The enzyme linked-immunosorbent assay has been widely used for the diagnosis of toxoplasmosis. Studies have been carried out to characterise and isolate *Toxoplasma* tachyzoite membrane, cytoplasmic antigen and somatic antigen (Sharma *et al.*, 1983; Ogata *et al.*, 1983; Decoster *et al.*, 1988, Bonhomme *et al.*, 1994). Both pellet and supernatant components of *Toxoplasma* tachyzoites have been used as antigens in various commercial diagnostic ELISA kits for toxoplasmosis (Jenum *et al.*, 1997) and immunoblot studies (Decoster *et al.*, 1988; Huskinson *et al.*, 1989; Santoro *et al.*, 1985). Supernatants of sonicated *T. gondii* tachyzoite suspension contain antigens referred to as exo-antigen (Bessieres *et al.*, 1992); these correspond at least partly to parasite secretions that play a role in parasite pathogenicity (Nichols *et al.*, 1983; Werk, 1985). We evaluated the use of these antigens in an in-house ELISA for the diagnosis of toxoplasmosis. This method was compared with that of the p-30 ELISA in which the membrane protein p-30, is used as antigen (Sharma *et al.*, 1983; Santoro *et al.*, 1985). The objective of the study is to develop an in-house ELISA for detection of IgG antibodies against *T. gondii* by using either supernatant and/or pellets of *Toxoplasma* tachyzoites as crude antigen. In addition, the antigenic structure of both antigens was studied by immunoblotting technique to determine the polypeptides recognised by IgG *Toxoplasma* antibodies in Thai patients.

Materials and Methods

Production of Toxoplasma antigen

Toxoplasma gondii RH strain was obtained from the Institute for Medical Research, Kuala Lumpur, Malaysia. *T. gondii* antigen was prepared as previously described (Ogata *et al.*, 1983; Mahakittikun *et al.*, 1998). Briefly, parasites were harvested from the peritoneal

cavity of Balb/c mice infected intraperironeally 3 days previously. Peritoneal fluid containing *Toxoplasma* was filtered through sterile glass wool to remove debris and host cells. The parasite suspension was collected and washed three times in PBS by centrifugation at 4°C (2,000 g for 20 minutes), and then resuspended in sterile distilled water. The suspension was sonicated for 5 minutes in an icebath and centrifuged again at 40,000 g for 20 minutes to separate the membrane antigen from soluble antigen. The pellets (P_1) consisting of surface membrane and cytoplasmic insoluble materials were collected separately from the supernatant fraction (S_1). The protein concentration of both parts were determined by the Pyrogallol Red method. The antigen was aliquoted to individual Eppendorf tubes and kept at -70°C until used as antigen in the ELISA. In immunoblot experiments, the antigens were prepared in the same manner except that the final pellet (P_1) was dissolved in 0.5% Nonidet P40 (Sigma) in PBS containing 100 u/ml of the protease inhibitor aprotinin (Sigma). After one-hour incubation at 4°C the extract was centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant (P_2) was used as the source of membrane antigen. The suspensions containing membrane antigen (P_2) and soluble antigen (S_1) were analysed by SDS-PAGE.

Test Sera

A total of 259 serum samples were collected from three subject groups as follows: Group I consisted of 69 serum samples from patients with symptoms suggestive of toxoplasmosis and positive *Toxoplasma* serology by immunofluorescent technique and Platelia Toxo-IgG ELISA (Sanofi diagnostics Pasteur). Group II consisted of 35 sera from patients presenting with different clinical conditions (5 SLE with positive anti-dsDNA, 10 hepatitis, 10 rheumatoid arthritis sera and 10 with other protozoan parasitic infections such as amoebiasis, giardiasis and with no serological evidence of toxoplasmosis). Group III consisting of 155 normal sera collected by random sampling from blood donors of the Department of Transfusion Medicine, Siriraj Hospital. All sera were kept at -40°C until tested for *Toxoplasma* IgG antibodies using ELISA and immunoblotting.

Control sera

The WHO international standard reference serum for human anti-*Toxoplasma* serum issued by Statens Serum Institute, Copenhagen, Denmark) and serum from a healthy person with negative *Toxoplasma* antibodies were used as the positive and negative control serum, respectively.

In-house ELISA

The assay was initially developed using polystyrene plates coated with pellets or supernatant antigen. The optimum concentrations of antigens, antibodies and conjugate used were determined on the basis of the best resolution obtained from positive and negative control sera. *Toxoplasma* antibodies were detected in the patient sera by sequential incubation as follows:

- 5% skim milk (100 µl, 5 min).
- diluted serum with 0.05% sodium azide in PBS-T (1:100, 100 µl, 30 min).
- goat antihuman IgG peroxidase conjugate in PBS-T, DAKO, Copenhagen, Denmark (1:5,000, 100 µl, 30 min).
- o-phenylendiamine substrate (Sigma) / H_2O_2/ citric acid-phosphate buffer, pH 5.0 (100 µl, 30 min).

Between the incubation steps, the samples were washed 5 times with PBS-T. The absorbance was read at 492 nm with a microplate reader (SLT ™). The cut-off value was determined using the mean plus twice standard deviation absorbance reading of healthy donors as the threshold.

P-30 ELISA (Platelia Toxo-IgG, Sanofi diagnostics Pasteur)

The test was carried out according to the manufacturer's instruction. Positive serum standard I (6 IU/ml), standard II (60 IU/ml), standard III (240 IU/ml) and negative control sera were included in each run in order to obtain a standard curve. Results expressed in IU/ml were derived from the standard curve, and sera with titres of IgG > 6 IU/ml was regarded as positive.

SDS-PAGE

The pellet (P_2) and the supernatant (SI) of *T. gondii* were each solubilized in sample buffer and electrophoresed in 13% polyacrylamide minigels (Miniprotean II, Bio-Rad) at 150 V, 0.25 A for 45 min. Twenty µg/ml of each antigen were applied per lane and visualised either by Coomassie Blue staining or transferred to nitrocellulose membranes. The prestained molecular-weight standards (Bio-Rad) were used as reference markers.

Immunoblot technique

The separated *Toxoplasma* peptides were transferred to nitrocellulose membranes using MiniTransblot (Bio-Rad) at 200 V, 0.12A for 60 min. After blotting the nitrocellulose sheet was incubated with 5% skim milk for 30 min to block any remaining protein binding sites. The nitrocellulose strips were cut and incubated overnight with rest serum diluted 1:50. After washing three times in PBS-T, the strips were incubated for one hour with 1:5,000 peroxidase labelled antihuman immunoglobulin (DAKO, Copenhagen, Denmark) and washed as above. To detect peroxidase activity, the strips were soaked for 5 min in the enzyme substrate (0.01 gm of 2,6 dichloroindophenol, 2 ml of 30% H_2O_2, 0.05M PBS pH 7.6) and washed with distilled water until the bands were clearly seen. Continuous shaking was maintained throughout the incubation period and washings.

Results

IgG antibodies to T. gondii in healthy persons

The in-house ELISA was used to determine the prevalence of *T. gondii* IgG antibodies in 155 serum samples from blood donors. One sample was found positive using antigen prepared from the pellet, and four samples were positive using antigens prepared from the supernatant. The positive cut-off values (mean + 2 S.D.) of the ELISA using pellets and supernatant as antigen were 0.60 and 0.55 respectively. Fig. 1 shows the mean and standard deviation of *Toxoplasma* IgG antibodies in various groups by in-house ELISA. Based on the cut-off level, a few samples in group II tested positive. Using Kappa analysis, we found that there was a good concordance between both antigens used in the in-house ELISA and p-30 ELISA with k coefficients = 0.869 and 0.822, $p < 0.001$ respectively.

In addition, results obtained in the in-house ELISA were studied further by immunoblot assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of both *T. gondii* tachyzoite antigens revealed more or less the same major antigenic peptide patterns, after staining with Coomasie blue. The antigens from pellet and supernatant showed 12 and 13 polypeptide bands respectively. They gave generally similar patterns but with different intensity. The antigenic bands which were recognised by IgG antibodies from the *Toxoplasma* seropositive sera were at 93, 66, 63, 58, 46, 35, 31 and 27 kDa. The intensely stained bands from both antigens ranged from 31 to 66 kDa. Fig. 2 shows the reactivity patterns between the antigens and test sera using Westernblot technique. Five of 69 sera from group I which had *Toxoplasma* IgM and also IgG antibody levels of more than 240 IU/ml, showed bands of 83, 66, 59, 33 and 31 kDa. Fifty-two of 69 (75.3%) sera from group I demonstrated a 31 kDa band, which was absent in all sera of the other groups. Bands of less than 21 kDa were not detected in the studied groups.

Discussion

We compared the results of our in-house ELISAs with p-30 ELISA, which uses membrane-enriched antigen of *T. gondii*. Our in-house ELISAs showed less specificity than p-30 ELISA. The disadvantage of the in-house

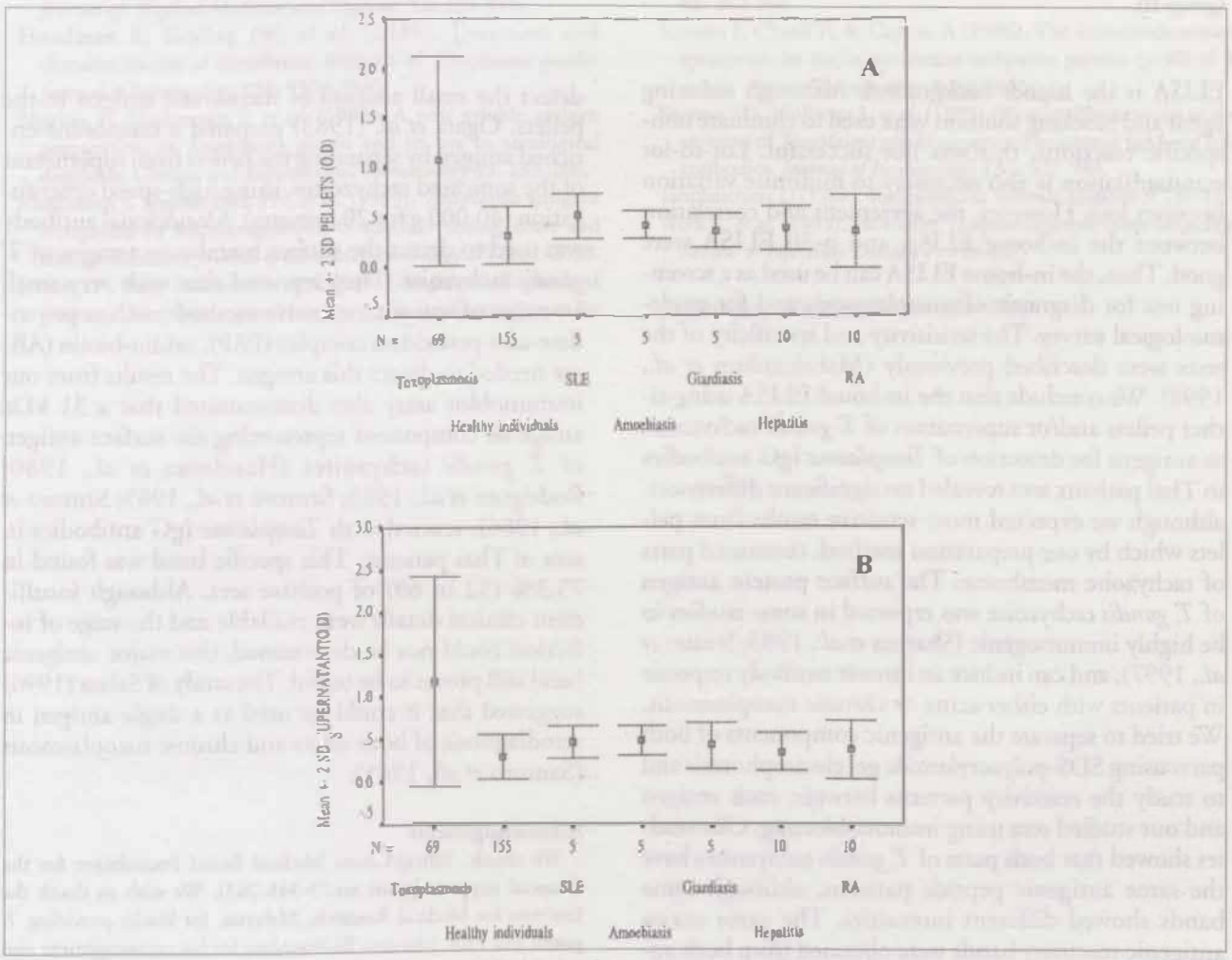

Fig. 1. Graph showing mean ± S.D. optical density (OD) values of *Toxoplasma gondii* IgG antibodies in various subject groups by in-house ELISA (A: using antigen prepared from pellet; B: using supernatant as antigen; see Materials and Methods for details).

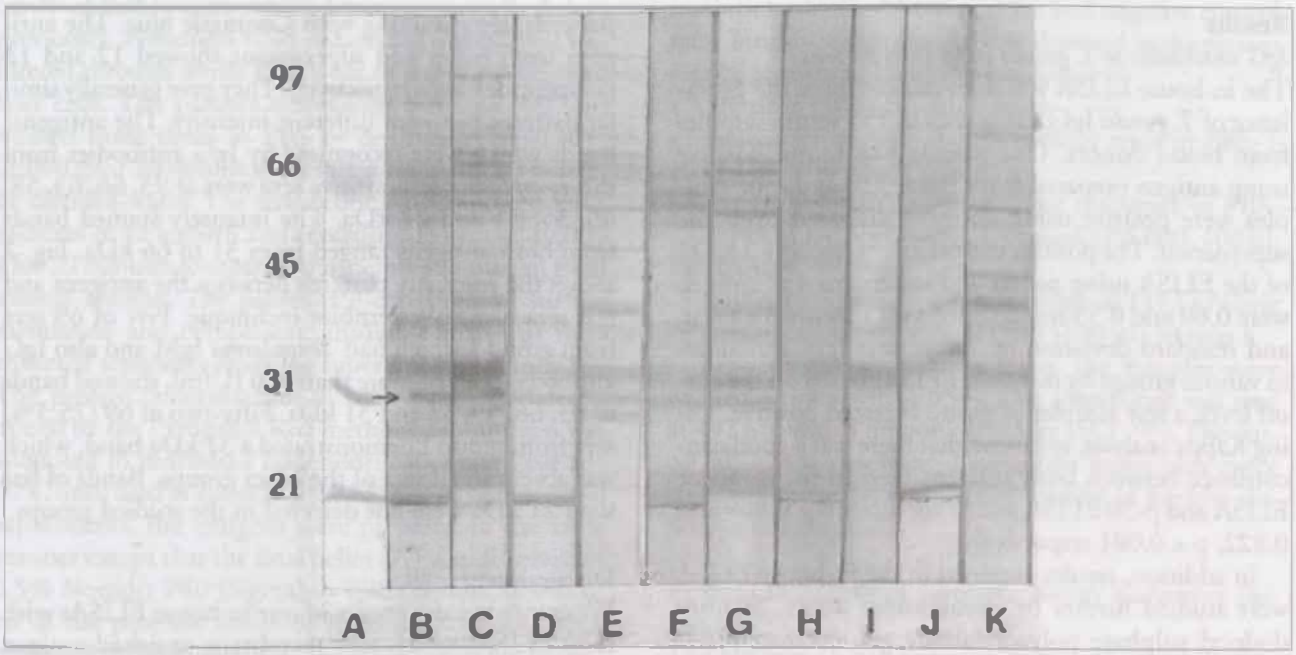

Fig. 2. Immunoblot pattern obtained with sera from different subject groups with *Toxoplasma gondii* antigens. S = supernatant antigen; P = pellet antigen (see Materials and Methods for details). Arrow indicates reactive polypeptide at 31 kDa. Lane A, standard markers; Lanes B & C, WHO positive refetence serum; Lanes D & E, serum from patient with acute toxoplasmosis; Lanes F & G, serum from patient with previous infection; Lanes H & I, pooled negative sera (group III); Lanes J & K, pooled sera from patients with other protozoan infections (group II).

ELISA is the higher background. Although reducing agent and blocking solution were used to eliminate non-specific reactions, this was not successful. Lot-to-lot standardisation is also necessary to minimise variation between lots. However, the agreement and correlation between the in-house ELISA and p-30 ELISA were good. Thus, the in-house ELISA can be used as a screening test for diagnosis of toxoplasmosis and for epidemiological survey. The sensitivity and specificity of the tests were described previously (Mahakittikun *et al.*, 1998). We conclude that the in-house ELISA using either pellets and/or supernatant of *T. gondii* tachyzoites as antigens for detection of *Toxoplasma* IgG antibodies in Thai patients sera revealed no significant differences, although we expected more sensitive results from pellets which by our preparation method, contained parts of tachyzoite membrane. The surface protein antigen of *T. gondii* tachyzoite was reported in some studies to be highly immunogenic (Sharma *et al.*, 1983; Jenum *et al.*, 1997), and can induce an intense antibody response in patients with either acute or chronic toxoplasmosis. We tried to separate the antigenic components of both parts using SDS-polyacrylamide gel electrophoresis and to study the reactivity patterns between each antigen and our studied sera using immunoblotting. Our studies showed that both parts of *T. gondii* tachyzoites have the same antigenic peptide patterns, although some bands showed diffetent intensities. The same major antigenic reactivity bands were obtained from both antigens as well. The results may be due to the inability to detect the small amount of membrane antigen in the pellets. Ogata *et al.* (1983) prepared a membrane-enriched antigen by separating the pellets from supernatant of the sonicated tachyzoites, using high-speed centrifugation (40,000 g for 20 minutes). Monoclonal antibody was used to detect the surface membrane antigen of *T. gondii* tachyzoite. They reported that with very small amounts of antigens, sensitive methods such as peroxidase-anti-peroxidase complex (PAP), avidin-biotin (AB) are needed to detect this antigen. The results from our immunoblot assay also demonstrated that a 31 kDa antigenic component representing the surface antigen of *T. gondii* tachyzoites (Handman *et al.*, 1980; Rodriguez *et al.*, 1985; Santoro *et al.*, 1985; Santoro *et al.*, 1986), reacted with *Toxoplasma* IgG antibodies in sera of Thai patients. This specific band was found in 75.3% (52 in 69) of positive sera. Although insufficient clinical details were available and the stage of infection could not be determined, this major antigenic band still proves to be useful. The study of Salata (1990) suggested that it could be used as a single antigen in serodiagnosis of both acute and chronic toxoplasmosis (Santoro *et al.*, 1985).

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