An Immunocytochemical Streptavidin-Biotin-Peroxidase-Complex Assay for the Diagnosis of *Toxoplasma* Infection

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ABSTRACT

An immunocytochemical streptavidin-biotin-peroxidase complex technique (SBC) has been developed for the serodiagnosis of toxoplasmosis and the overall reliability of the developed technique was evaluated by comparing the results with that of immunofluorescent assay (IFA) and enzyme linked immunosorbent assay (ELISA). These were good agreement between qualitative results on all tests (kappa analysis, k=0.87 for SBC and ELISA; k=0.87 for IFA and ELISA). Pearson correlation analysis revealed a good correlation in quantitative results on all tests as well (r=0.714 and p<0.001 for SBC and ELISA; r=0.683 and p<0.001 for IFA and ELISA; r=0.775 and p<0.001 for SBC and IFA). The SBC is easy to perform under limited laboratory facilities and under field conditions, thus it is likely to be another tool for routine diagnosis and field survey of *Toxoplasma* infections that is both sensitive and effective.

INTRODUCTION

Toxoplasma gondii has been recognized as an important cause of morbidity and mortality among immunocompromised persons. It is one of the most frequently encountered opportunistic infections in patients with the acquired immune deficiency syndrome (AIDS) (Holliman, 1988). Toxoplasmosis can be life-threatening in congenitally infected children as well (Lin et al. 1995).

The diagnosis of *T. gondii* infection is currently based on classical

immunological tests (Foudrinier et al. 1995), although new and advanced molecular biotechnological techniques has been recently introduced as potentially new tools in the diagnosis of *T. gondii* infection and other parasitic infections (Bucton 1993). This current diagnostic technique may be adequate, sensitive and rapid but in developing nations the necessary equipment and reagents used in these new and advanced techniques are often unavailable. In some laboratories, even a fluorescent microscope is too expensive to obtain. By the year 2000, WHO projected that 26 million people will be infected with HIV, more than 90% of whom will be in developing countries (Quinn 1996). Therefore, a suitable serologic test for diagnosing toxoplasmosis and which can also be used in seroepidemiological surveys of toxoplasmosis should be proposed.

Immunocytochemical techniques using enzyme-conjugated reagents which produce a color reaction has proven to be powerful tools for the accurate diagnosis of a number of diseases (Taylor 1978; Falini and Taylor 1983). Since these techniques produce colored products that are permanent and intense and which can be viewed with the light microscope (Jones and Gregory 1990), it is thus suitable for laboratories where fluorescent microscopes or equipment used in carrying out the ELISA technique are unavailable.

In this study, the immunocytochemical streptavidin-biotin-peroxidase complex (SBC) assay was developed for the serodiagnosis of toxoplasmosis and the overall reliability of the developed technique was evaluated by comparing the results with that of the immunofluorescent assay (IFA) and enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Antigen

Toxoplasma gondii RH strain was harvested in phosphate buffered saline (PBS), pH 7.2, from the peritoneal exudate of mice infected 3 days earlier with the parasites. They were washed 3 times in PBS by centrifugation at 600g for 10 min and examined under the microscope. Only samples that contained less than 5% white blood cells were selected for resuspending to 2×10^7 trophozoites/ml. The suspension was coated on Teflon slides and fixed with absolute ethyl alcohol for 10 min. The slides were then kept at -70°C until used in IFA and SBC techniques.

Serum samples

Two hundred sixty serum samples used in this study were obtained from Department of Clinical Pathology, Department of Parasitology and Department of Blood Bank, Siriraj Hospital, Bangkok, Thailand. All serum samples were tested

for the presence of IgG to *T. gondii* by a commercial ELISA kit (Platelia® Toxo IgG) and were classified into groups according to their serologic status in relation to *T. gondii* infection as follows:

1. 60 serum samples were from cases who were diagnosed with toxoplasmosis on the basis of clinical, pathological and laboratory evaluation.
2. 20 serum samples were from cases with other parasitic infections, patients with other diseases such as rheumatoid arthritis, SLE (diagnosis of these diseases made on the basis of clinical, laboratory and pathological evaluation) and patients who were suspected of having toxoplasmosis but have no *Toxoplasma* antibodies.
3. 180 serum samples were from healthy blood donors, collected by random sampling method.

All serum samples were kept at -70°C until used in a comparison study between the SBC, IFA and ELISA.

Control serum samples

An international standard reference serum containing 1,000 International Units/ml (IU/ml) of IgG antibodies to *T. gondii* (provided by WHO at Statens Serum Institute, Copenhagen, Denmark) was used as positive control serum and serum from healthy person with negative *Toxoplasma* antibodies was used as negative control serum in IFA and SBC assays.

Indirect immunofluorescent assay (IFA)

The indirect immunofluorescent technique was performed according to the method of Chessum (1970). For each run, positive, negative and PBS controls were included. The titers are expressed in International Units per ml of serum according to WHO recommendation, with reference to an international standard reference serum (WHO 1968).

Immunocytochemical 'Streptavidin-Biotin-Peroxidase Complex' assay

The antigen slides were left at room temperature for 5 min and the serum dilutions dispensed into designated wells. Following 30 min incubation at 37°C, the slides were washed twice at 4 min each, after which the anti-human IgG-biotin (DAKO) was added to each well and then allowed to react for another 30 min at 37°C. The slides were then washed as above. An optimal dilution of Streptavidin-biotin-peroxidase complex (dilution 1:200) was dispensed into each well and then incubated for another 30 min at 37°C, after which the slides were washed again

and a substrate solution containing 25 mg of 3',3-Diaminobenzidine tetra-hydrochloride in 100 ml of 0.01 M Tris-HCl buffer, pH 7.2, 200 μl of 3% H_2O_2 was added and allowed to react at room temperature for 10 min. The slides were rinsed with PBS and mounted in 70% (v/v) glycerol in PBS for microscopic examination ($\times$40 objective). For each run, positive, negative and PBS controls were included. All samples were observed by the same investigator without prior knowledge of the serologic test results of the samples.

the titers are expressed in International Units per ml of serum, in referenced to an International standard reference serum. If the International standard reference serum titrated to 1,000 IU/ml still yields a positive reaction at 1:2,048 dilution, the patient's serum which is positive at dilution 1:512 is given a titer of 250 IU/ml.

Platelia® Toxo IgG (Sanofi diagnostic pasteur)

This indirect enzyme-linked immunosorbent assay was carried out on eight-well strips coated with *Toxoplasma* antigen (enriched with membrane antigen P-30). The detailed method was performed following the manufacturer's instructions. Positive serum standard I (6 IU/ml), standard II (60 IU/ml), and standard III (240 IU/ml) plus a negative control serum sample were included in each run in order to obtain a standard curve. The optical densities (OD) were read using an ELISA reader. Results expressed in IU/ml were derived from the standard curve and serum samples with IgG titers above 6 IU/ml were regarded as positive.

RESULTS AND DISCUSSION

A total of 260 serum samples were evaluated in a comparison study using the SBC, IFA and ELISA. In the SBC assay, tachyzoites of *T. gondii* stained brown to indicate a positive reaction while they remained colorless in a negative reaction (Fig. 1). The end point was taken to be the last serum dilution that still stained brown. Although IFA was found to be simpler and less time consuming to perform taking approximately 1.5 hrs while the modified SBC took about 2.5 hrs, special equipment is required for the test and the IFA preparation is not permanent due to the fading of fluorescence. Some advantages of the SBC test are that it produces a permanently stained slide and thus provides a long-term record of the results, and is cost effective since no special equipment are required for the test. Indirect immunoperoxidase technique (IIP) has also been used in many immunodiagnostic procedures but development of the avidin-biotin-peroxidase technique resulted in a higher sensitivity (Hall and Ward 1984). It was found that a maximum signal to background ratio was obtained by substituting avidin with
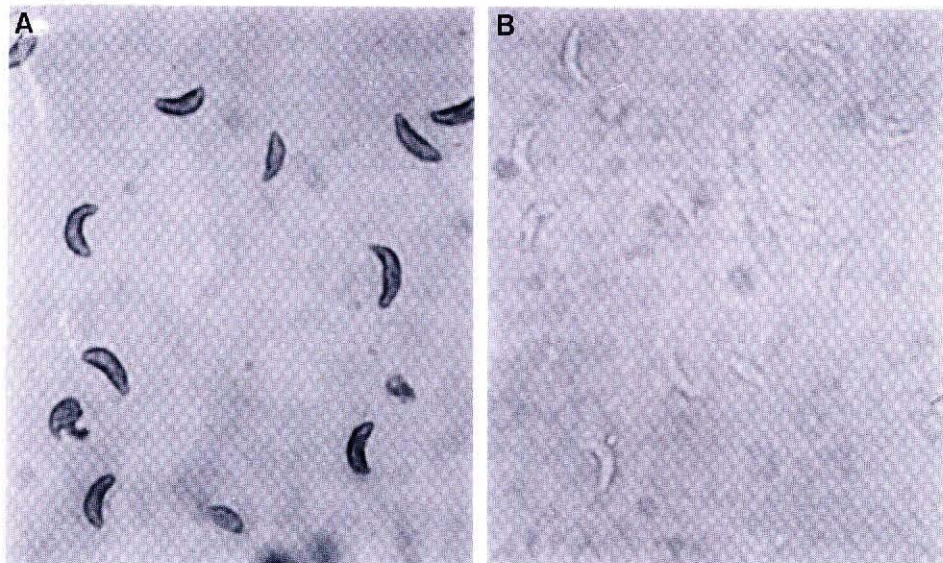
Fig. 1 The SBC staining pattern for the detection of antibodies to *Toxoplasma gondii* (×400). A: positive serum reaction with tachyzoites, B: negative serum reaction.

streptavidin which resulted in reduced non-specific background staining (Wood and Warnke 1981), as well as by optimizing the amount of substrate DAB used which also effectively reduced non-specific background staining, although a protein blocking step was omitted in this study. King et al. (1997) demonstrated in his experiment that there were no visible differences in the final immunohistochemical results whether or not a protein blocking step was included since omitting any protein blocking step did not produce any background staining.

Table 1 Correlation of positive and negative results given by SBC, IFA and ELISA[R] in the detection of antibodies to *Toxoplasma gondii*.

ELISA	SBC[a]			IFA[b]		
	+	-	Total	+	-	Total
+	55	5	60	54	6	60
-	7	193	200	6	194	200
Total	62	198	260	60	200	260

a: k=0.87, b: k=0.87.

Table 1 summarizes the concordance among the results obtained from three assays calculated by kappa analysis. Qualitatively, there was good concordance between the IFA, SBC and ELISA (k=0.87 for SBC and ELISA; k=0.87 for IFA and ELISA). From Table 2, sensitivity and specificity of SBC are 91.6% and 97.0% respectively when using ELISA as the gold standard, and SBC has 100% sensitivity and 99% specificity when IFA was used as the gold standard.

Table 2 Performance of SBC compared to IFA and ELISA.

SBC	vs IFA	vs ELISA
% sensitivity	100% (60/60)	91.6% (55/60)
% specificity	99.0% (198/200)	97.0% (193/200)
% predictive value	88.0% (55/62)	90.0% (54/62)

In this study, the results from all assays were expressed in International Units (IU/ml). For IFA and SBC, the results in titer were converted to IU by comparing the values against the International standard reference serum provided by WHO, and those with a cut-off value as a titer above 8 IU/ml. For the ELISA, the positive control sera were available in the kit and interpretation in IU/ml are calculated according to the manufacturer's instructions. For quantitative evaluation, a positive result using the three assays was determined by Pearson's Correlation analysis which revealed that there is a good correlation (r=0.714 and p<0.001 for SBC and ELISA; r=0.683 and p<0.001 for IFA and ELISA; r=0.775 and p<0.001 for SBC and IFA), although there are some statistically significant differences in quantitative evaluation between IFA and ELISA (Paired t-test: p<0.01), and between the SBC and ELISA (Paired t-test: p<0.01). On the contrary, there was no statistically significant difference quantitatively between the IFA and SBC (Paired t-test: p<0.05). Petithory et al. (1996) commented that expression of the results in IU/ml has the advantage of presenting everything in a standard scale along with various other tests, but caution is required since the definition of IU varies according to the test and reagents used.

For serum samples with low concentrations of *Toxoplasma*-specific IgG antibodies, the IFA and SBC yielded some negative results, which also detected a lower level of the specific IgG antibody when compared to the ELISA. This result confirmed the previous study which reported that ELISA correlated well with IFA but is generally more sensitive than the IFA in detecting *T. gondii* antibody levels

(Chan et al. 1985).

This study has demonstrated that the SBC is as effective as the IFA in measuring antibody levels to *T. gondii* and that there is a good correlation between both tests. However, the SBC seems to be more sensitive quantitatively than the IFA, since among 50 serum samples positive by both tests, 27 samples (54%) detected the same antibody titer. Among the rest, 7 serum samples (14%) revealed a lower antibody titer than the IFA while 20 serum samples (40%) gave a higher antibody titer.

Similar observation was made by Bracho and Perez (1992), Gentillini et al. (1975), and Lim (1988) who all utilized the immunoperoxidase technique in diagnosing some parasitic diseases. Lim (1988) suggested that the greater light intensity of the bright field system probably gave a more accurate evaluation of the color reaction than with the less intense fluorescence system, especially at dilutions approaching the end point.

In terms of cross reactivity, the 30 serum samples did not yield false positive reactions with other diseases and other parasitic infections. It is recommended that the specific anti-*Toxoplasma* IgM antibody be evaluated with this immunocytochemical technique. Unfortunately, no serum sample obtained in this study possess IgM antibodies to *T. gondii*.

In conclusion, the SBC is easy to perform under limited laboratory facilities and under field conditions, thus it is likely to be another sensitive and effective tool for the routine diagnosis and field survey of *Toxoplasma* infection.

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