# A Simple and Economic Slide Micro-Immunoenzymatic (Micro-SIA) Test for Epidemiological Studies of Toxoplasmosis

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*A slide micro-immunoenzymatic assay (micro-SIA) to detect antibodies to non-particulate* Toxoplasma gondii *antigens is described. This assay allows the diagnosis of toxoplasmosis infection in about 1 hr. Twenty-four determinations can be performed per slide. Five hundred ng of antigen and 5 or 10 µl drop of each reactive are necessary per well. The clear contrast of colours obtained for negative and positive sera after the test is finished, allows direct discrimination of the results. However, it is possible to quantify the results of the reaction using a minireader. Sera dilution cutoff value, determined as the most frequent titre for the general population, is 1:100. The toxoplasma micro-SIA correlates well with indirect immunofluorescence (IIF), its sensitivity is at least three times as much as IIF. The test has an intra and inter assay variation coefficient of 5.46% and of 6.24% respectively. Sera obtained at random from argentinian people were analyzed and a 56% of infection was found. The main features of the Toxoplasma micro-SIA are its simplicity, sensitivity, reproducibility, and the virtual absence of background making it very suitable for screening tests.*

Key words: toxoplasmosis – serodiagnosis – microELISA – epidemiology

Toxoplasmosis is one of the most frequent and worldwide dispersed infections in humans (Frenkel 1971). Most of the time the absence of clear symptoms requires a serological assay for a correct diagnosis. The severe consequences that the acute stage of the disease produces on fetuses and neonates (Remington & Desmonts 1983) indicate a preventive screening test in pregnant women.

Indirect hemagglutination (IHA) is the most utilized system in Argentina and other countries. Indirect immunofluorescence (IIF) is another system widely used. The first (IHA) is very simple but often leads to false positive results, consumes a great quantity of antigen and often recent infections are not detected (Tomasi et al. 1986). IIF is sensitive and rapid, but immunofluorescent microscopy and "trained eyes" are necessary, resulting in important difficulties for the assay automatization. Enzyme-linked immunosorbent assay (ELISA) is the other alternative system for toxoplasmosis serodiagnosis (Ambroise-Thomas & Desgeorges 1980, Tomasi et al. 1986). It is very simple and has sensitivity levels similar to IIF.

The aim of our work was to design a simple and economic test for immunodiagnosis of toxoplasmosis useful in clinical laboratories and that could be applied in epidemiological studies. Micro-SIA was found to be more sensitive and reliable than IIF and ELISA due to the virtual absence of background (Conway de Macario et al. 1983). On the other hand, it is very simple, rapid and requires small amounts of sera, antisera and reagents. In view of this advantages, we have designed a slide micro-immunoenzymatic assay (micro-SIA), with non-particulate antigens for immunodiagnosis of toxoplasmosis.

## MATERIALS AND METHODS

*Antigen* – Tachyzoites of *T. gondii*, RH strain, harvested from mouse peritoneal exudate, 72 hr after infection, were resuspended in phosphate-buffered saline (PBS), pH 7.4 with the addition of EDTA, aprotinin and phenylmethylsulfonyl fluoride (PMSF) at final concentrations of 2.5mM, 100 units/ml and 0.1 mM respectively, and frozen and thawed three times. The soluble extract (1.5 mg of proteins/ml) was diluted in PBS to make the desired antigen dilution and was used to sensitize the immunoassay slides.

*Sera* – Control sera (classified by results of immunofluorescence and indirect hemagglutina-

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tion) were used at appropriate dilutions. In addition, 448 serum samples were collected from Buenos Aires city and its suburbs people. Samples were decomplemented by heating 30 min at 56°C and stored at –20°C until use. For testing these sera, serial dilutions were made in PBS, pH 7.4, 1.5% non fat milk. A standard WHO serum was used to determine the sensitivity of micro-SIA and IIF tests.

*SIA procedure* – The assay presented here is a modification of the one described by Conway de Macario et al. (1983). Briefly, slides with 24 circles (Cel Line Associates, Inc., New Jersey) were cleaned with 95% (v/v) ethanol. Five µl of the antigen dilution were loaded on each micro-SIA well and air-dried. Antigen coated slides were washed in distilled water, blocked, washed again in distilled water and air-dried. The air-drying procedure could be shortened by using a Titan micro hood (Helena Laboratories, Beaumont, TX). Then, 5 µl of serum dilution were placed on each circle and slides were kept at room temperature in a humid chamber, for 20 min. Circles were then washed three times in PBS and incubated with 5 µl of peroxidase conjugated rabbit immunoglobulins to human IgA, IgG and IgM from Dako, Denmark diluted 1:1000 for 20 min. Serum and second antibody were diluted in PBS, pH 7.4, 1.5% non fat milk. The wells were washed three times in PBS for 5 min each and once in distilled water for 1 min after each incubation. Slides were air-dried as described above prior to the addition of 10 µl of the substrate solution (o-phenylenediamine dihydrochloride in 0.1M citrate buffer, pH 4.5 containing 0.15% of 30% $H_2O_2$ (v/v), to obtain a 1mg/ml solution). After a further 15 min incubation at room temperature in the presence of substrate, 10 µl of 1N sulfuric acid were added and the absorbance read at 420nm in a minireader MR590 (Dynatech Instruments, Alexandria, VA). Sera dilution cutoff value, determined as the most frequent titre for the general population, was 1:100.

*IIF procedure* – Parasites for IIF test were obtained as described above and then were purified by one cycle of centrifugation in a sucrose gradient (Garberi et al. 1990), washed three times in PBS, and fixed in 1% formalin during 30 min at room temperature. IIF test procedure, reactives and equipment were the same as those used by the "Instituto Nacional de Microbiologia Carlos G. Malbran". Briefly, about 50 fixed parasites per microscope field were loaded in each well. Then, slides were air-dried and washed in distilled water. Each well was incubated with 20 µl of serum dilution for 30 min. After washing twice in PBS, 20 µl of fluorescein conjugated anti-human IgG from Dako, Denmark diluted 1:200 in PBS were placed on each circle and incubated for 30 min. Slides were washed again in PBS and examinated under Carl Zeiss fluorescence microscopy with a low power (12 V) halogen lamp. The validity of this test was confirmed by comparison with Sabin-Feldman assay which was calibrated to IU/ml with reference to WHO standard.

*Regression analysis* – To compare micro-SIA with IIF each human sera, in total 448, was titrated by both methods by successive 1:2 dilutions, starting from 1:12.5 for micro-SIA and from 1:4 for IIF assay. Regression analysis was performed considering the logarithm of micro-SIA and IIF titres as independent variables.

*Evaluation of epidemiological data* – To determine the positivity or negativity of the reaction each human sera was assayed at a dilution correspondent to the cutoff value by both techniques. For micro-SIA assay the reaction was considered positive when the absorbance obtained was higher than 0.01. Contingency tables were constructed and the chi square test was performed in order to determine significant differences in toxoplasmosis prevalence among people harbouring physiological (pregnancy) or pathological (allergy, rheumatism, cancer, endocrinological anomalies, HIV and *Trypanosoma cruzi* infections) alterations, and a healthy control group. People selected for this analysis were between 30 to 55 years old.

## RESULTS

In order to find the adequate antigen mass to perform Toxoplasma micro-SIA, different quantities of *T. gondii* antigen were loaded in the wells of the slide. For each antigen quantity, the assay was performed using different serum dilutions, i.e.: 1:100; 1:200 and 1:400 (Fig. 1). The largest differences between the absorbances of positive and negative control sera were obtained with antigens levels of 500 and 700 ng of *T. gondii* protein. We chose 500 ng of antigen mass because practically no absorbance was obtained for the negative control serum, even at 1:100 dilution.

Experiments were performed with different washing and blocking conditions. It was found that Toxoplasma micro-SIA had better results when washings were carried out in PBS. The blocking agent chosen was non fat milk diluted in PBS in a final concentration of 1.5%. The optimum blocking time was found to be 40 min, because it produced the higher difference in absorbance between negative and positive control serum (Fig. 2).

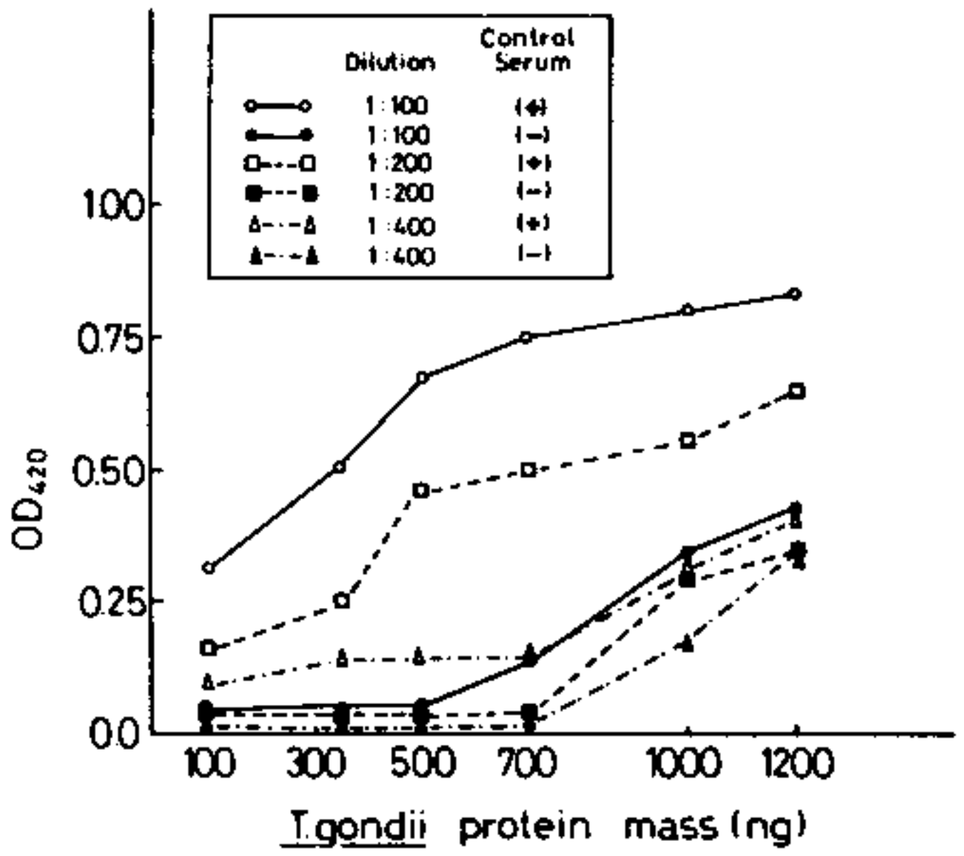


Fig. 1: effect of antigen quantity (expressed as *Toxoplasma gondii* protein mass) on absorbance readings obtained after performing micro-SIA assay with positive and negative control sera, at three sample dilutions.

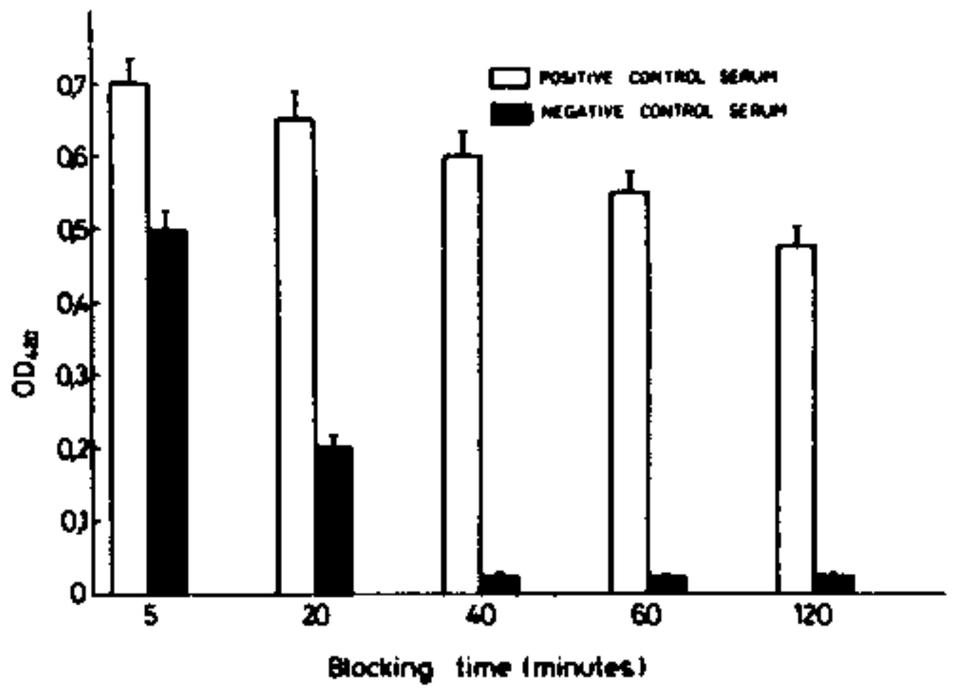


Fig. 2: effect of blocking time on absorbance readings ($\bar{X}$ ± SD) obtained after performing micro-SIA assay with positive and negative control sera.

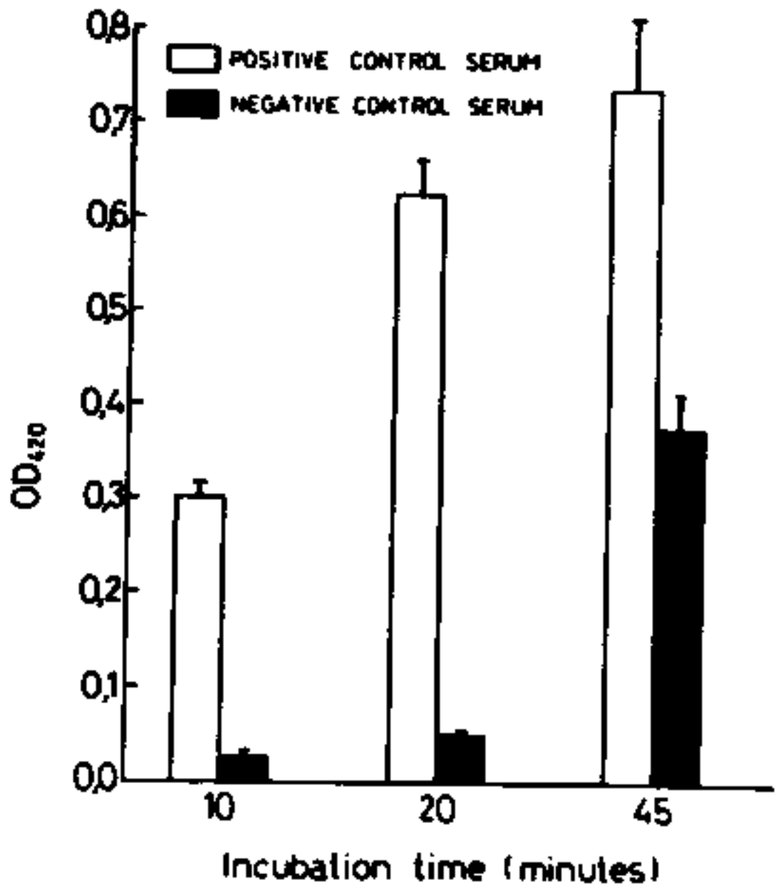


Fig. 3: effect of serum incubation time on absorbance readings ($\bar{X}$ ± SD) obtained after performing micro-SIA assay with positive and negative control sera.

On the other hand, the best conditions for the incubation with serum were 20 min at room temperature (Fig. 3). Longer incubations resulted in higher absorbances, but the resolution observed between positive and negative sera was not as good as for 20 min. Shorter incubations had similar problems, accompained by a very low absorbance.

Experiments designed to study the stability of antigen coated and blocked slides gave a shelf life at 4 °C of at least one year (data not shown).

Once the assay conditions were established, analyses were made to determine if micro-SIA is appropriate for toxoplasmosis diagnosis. Micro-SIA and IIF toxoplasmosis titres were determined in 448 sera obtained from Buenos Aires city and its suburbs (Argentina) people selected at random. Titre was defined as the inverse of the highest serum dilution giving an absorbance at 420 nm higher than 0.01. A good correlation was found between micro-SIA and IIF titres ($r = 0.855$) (Fig. 4).

The sensitivity of Toxoplasma micro-SIA, determined with a WHO control serum, resulted in 3 IU/ml at 1:100 serum dilutions (data not shown). At the same time the sensitivity of IIF test was 10 IU/ml at 1:32 sera dilution. Therefore, this test resulted more sensitive than IIF. In coincidence with this result, for all the sera tested micro-SIA titres were higher than IIF titres (Fig. 4). In addition, 206 sera (out of 448) which had low IIF titres (32 or 64) were analyzed by micro-SIA. Thirty-three percent of these sera gave titres of 200 or 400, 61.2% gave titres of 100 and only 5.8% gave titres of 50 (data not shown).

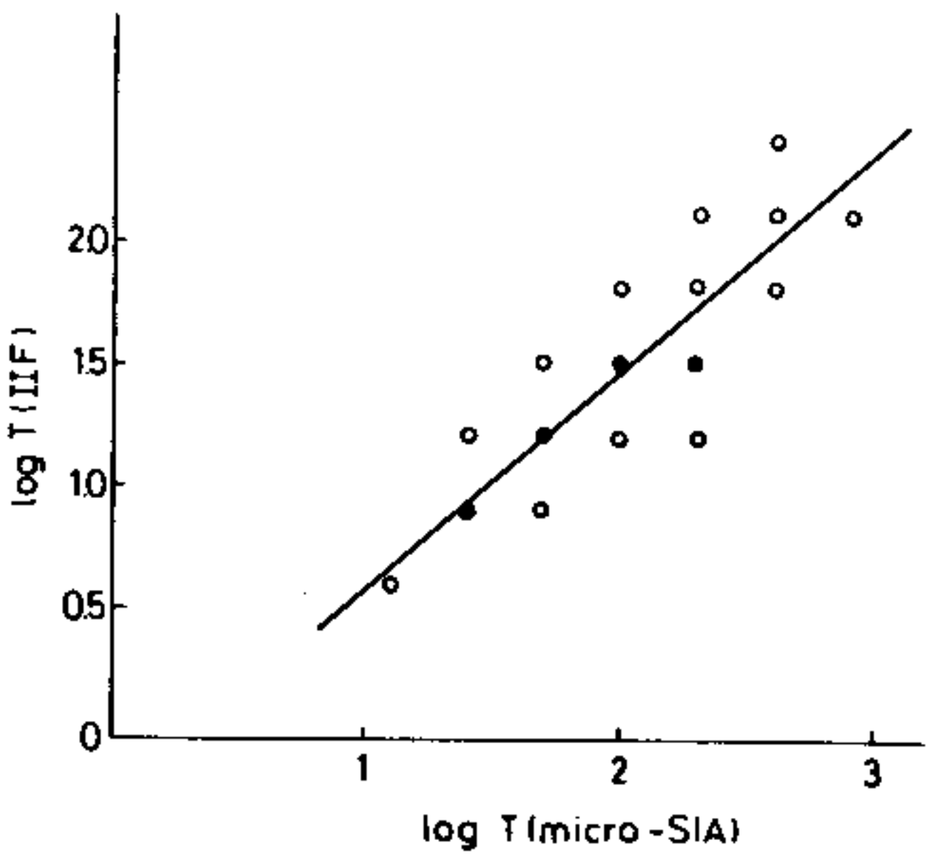


Fig. 4: correlation between micro-SIA and IIF titres of 448 sera samples. Each point represents more than one serum sample, $n > 36$ (filled circles) or $n < 36$ (open circles). The calculated regression line is shown.

TABLE I

Contingency table to determine independence in prevalence of toxoplasmosis positive titres among people harbouring physiological or pathological alterations and controls

| People group | micro-SIA | | IIF | |
|---|---|---|---|---|
| | No. of positive sera | No. of negative sera | No. of positive sera | No. of negative sera |
| Pregnancy | 34 (50.0%) | 34 (50.0%) | 34 (50.0%) | 34 (50.0%) |
| Allergy | 20 (50.0%) | 20 (50.0%) | 20 (50.0%) | 20 (50.0%) |
| Rheumatism | 18 (45.0%) | 22 (55.0%) | 18 (45.0%) | 22 (55.0%) |
| Cancer | 24 (57.1%) | 18 (42.9%) | 23 (54.8%) | 19 (45.2%) |
| End. Anom. | 31 (51.7%) | 29 (48.3%) | 31 (51.7%) | 29 (48.3%) |
| HIV | 24 (50.0%) | 20 (50.0%) | 22 (45.8%) | 26 (54.2%) |
| Chagas | 14 (43.8%) | 18 (56.2%) | 14 (43.8%) | 18 (56.2%) |
| Control | 34 (56.7%) | 26 (43.3%) | 32 (53.3%) | 28 (46.7%) |

End. Anom.: endocrinological anomalies.
Number between brackets indicates percentage of positive and negative sera for each group.

To determine the reproducibility of the method, one control serum was assayed 20 times in the same slide and 20 times in different assays. The intra assay variation coefficient resulted 5.48% and the inter assay variation coefficient was 6.24%.

The interference of the method with other physiological (pregnancy) or pathological (allergy, rheumatism, cancer, endocrinological anomalies and HIV or *T. cruzi* infections) was analyzed. Chi square test indicated that there are no significant differences in percentage of Toxoplasma micro-SIA positive results among physiological or pathological alterations above mentioned and healthy control people. The same results were obtained for IIF assay (Table I).

Four hundred and forty eight sera were analyzed to determine the prevalence of toxoplasmosis in Buenos Aires city and its suburbs (Table II). Positive serology was 56.3% (micro-SIA) or 54% (IIF).

TABLE II

Prevalence of toxoplasmosis in Buenos Aires and Buenos Aires suburbs (Argentina) determinated by micro-SIA or IIF assays

| Technique | No. of positive sera | No. of negative sera |
|---|---|---|
| micro-SIA | 252 (56.3%) | 196 (43.7%) |
| IIF | 242 (54.0%) | 206 (46.0%) |

Number between brackets indicates percentage of positive and negative sera for each group.

## DISCUSSION

There are various immunodiagnosis systems to detect toxoplasmosis: Dye test, IIF, IHA, ELISA, and IgM specific tests (Sabin & Feldman 1949, Walton et al. 1966, Tomasi et al. 1986, Duffy et al. 1989). Because of the complexity of the immune response, it is recommended to perform more than one assay. Sensitivity and simplicity of the tests are two important features required. We report here a micro-ELISA system (named slide micro-immunoenzymatic assay) for Toxoplasma serology.

Micro-SIA system showed a high sensitivity, three times as much as IIF. Also, it resulted more sensitive than ELISA, for which a sensitivity of 8 IU/ml was reported (Joss et al. 1989). It is also very simple, rapid and economic, because it required little amounts of samples and reagents (5-10 µl). No special laboratory equipment is required and results can be assesed by naked eye because of background absence. Antigen coated slides could be stored at 4°C for at least one year. This allowed the preparation in advance of a stock of slides and to perform several determinations very rapidly (about 1 hr).

The correlation found between IIF and micro-SIA was very good ($r = 0.855$), indicating that this assay could be an alternative to the indirect immunofluorescent test which requires a fluorescence microscopy and technical training.

Recently, recombinant *T. gondii* antigens useful for serodiagnosis were obtained (Gross et al. 1992, van Gelder et al. 1993). We consider that Toxoplasma micro-SIA may be used with these antigens.

Furthermore, no interference or false positivity was found with sera obtained from people harbouring other physiological or pathological alterations.

All the features stated above make Toxoplasma micro-SIA very suitable for epidemiological studies and allowed us to analyze the prevalence of toxoplasma infection in samples obtained at random from Buenos Aires city and its suburbs (these two places concentrate 40% of argentinian population). The high prevalence of toxoplasmosis seropositivity found in this study (55-56%) is in good agreement with reports from other countries around the world like France (55%), Holland (58%), Italy (60%) and Austria (62%), (Remington & Desmonts 1983) confirming the worldwide dissemination of the disease.

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