

Use of three immunological techniques for the detection of *Toxoplasma* spIgA antibodies in acute toxoplasmosis

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Abstract

Aims—To assess the performance and efficacy of three immunological techniques for the detection of *Toxoplasma* specific IgA antibodies in acute toxoplasmosis.

Methods—The following techniques were used to examine 128 serum samples (51 cases of acute toxoplasmosis, 50 cases of heterologous infections, and 27 healthy controls): direct enzyme linked immunosorbent assay (ELISA), antibody capture ELISA, and antibody capture agglutination.

Results—Direct ELISA had a sensitivity of 98% and a specificity of 97%, antibody capture ELISA of 100% and 99%, respectively, and antibody capture agglutination had sensitivity and specificity of 100%.

Conclusions—All three immunological techniques performed well with similar efficacy. Detection of *Toxoplasma* specific IgA antibodies is a useful diagnostic marker for acute toxoplasmosis.

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Toxoplasmosis, a ubiquitous protozoan infection caused by an intracellular parasite, *Toxoplasma gondii*, is generally asymptomatic or is associated with mild non-specific clinical symptoms in immunocompetent subjects.^{1,2} The parasite can, however, cause serious illness in congenitally infected infants^{3,4} and in immunocompromised patients.⁵⁻⁷ Serological diagnosis of acute toxoplasmosis has traditionally been made by detecting specific IgM antibodies or by the demonstration of a significant increase in specific IgG antibody levels, or both. However, because of the prevalence of high IgG *Toxoplasma* antibody titres in normal subjects and because, in some people, specific IgM antibodies can persist for several months or even years following the acute infection, the interpretation of serological test results is difficult when acute toxoplasmosis is suspected.

Several reports have emphasised the value of the detection of *Toxoplasma* specific IgA antibodies for the diagnosis of acute human toxoplasmosis.⁸⁻¹² The aim of the present study was to assess the performance and efficacy of three immunological techniques for the detection of *Toxoplasma* specific IgA antibodies in acute toxoplasmosis. The first technique was a quantitative direct enzyme linked

immunosorbent assay (ELISA) developed in our laboratory, using plates coated with soluble antigen from sonicated *T gondii*. The second was a commercial quantitative ELISA technique based on the antibody capture sandwich principle (ETI-TOXOK-A reverse, Sorin Biomedica, Saluggia, Italy). The third technique was based on the antibody capture agglutination principle.^{8,10}

Methods

Unless otherwise stated, the chemicals and reagents used in this study were obtained from the Sigma Chemical Co., St Louis, Missouri, USA.

All serum samples (n = 128) included in this study were obtained from patients seen at the University Hospital of the State University of Campinas (Campinas, São Paulo, Brazil). Of the 128 serum samples, 51 were from patients with serological evidence of acute toxoplasmosis. *Toxoplasma* specific IgM antibodies were detected in these patients using both an antibody capture ELISA (Sorin Biomedica, Italy) and an indirect fluorescence antibody test (IFT). *Toxoplasma* specific IgG antibodies were also detected using IFT. A significant increase in the titre of such antibodies was detected in 16 of 51 patients. The serum samples were received for testing at our laboratory because the patients were suspected of having an acute toxoplasmosis infection. All 51 patients had lymphadenopathy. The groups of nodes most commonly involved were, in order, the cervical, axillary, and inguinal. Forty three patients (84%) had cervical lymphadenopathy.

Seven serum samples were obtained from patients with cytomegalovirus (CMV) infection, in whom CMV specific IgM antibodies were detected using an antibody capture ELISA (Sorin Biomedica, Italy). Ten serum samples were obtained from patients with rubella virus infection. Rubella specific IgM antibodies were also detected using an antibody capture ELISA (Sorin Biomedica, Italy). Eight serum samples were from patients with infectious mononucleosis, a diagnosis based on the results of heterophile antibody tests. Ten serum samples were from patients with hepatitis B virus (HBV) infection, in whom both hepatitis B surface and core antigens were detected on ELISA (Sorin Biomedica, Italy). Five serum samples were from patients with hepatitis A virus (HAV) infection, in whom HAV specific IgM antibodies were detected on ELISA (Abbott, Illinois, USA).

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Six serum samples were from patients with syphilis, which were positive on the VDRL and TPHA tests. Four serum samples were from patients with Chagas' disease, with *T cruzi* specific IgG antibodies on both IFT and complement fixation tests.

Twenty seven serum samples from healthy students and laboratory personnel with no clinical or serological evidence of recent infection by *T gondii* served as controls.

DIRECT ELISA

Tachyzoites of the RH strain of *T gondii*, obtained from the peritoneal cavity of Swiss mice two days after infection, were washed four times by being centrifuged with sterile phosphate buffered saline (PBS), pH 7.2. The final parasite pellet was resuspended in PBS containing 0.25 mM phenylmethylsulphonyl fluoride to about 10 times the pellet volume. The tachyzoite suspension was sonicated in an iced water bath using a Branson sonicator (model SX-30B, Branson Ultrasonics, Danbury, Connecticut, USA), at a power setting of three with a 20% pulse duty cycle, for three minutes. The sonicated suspension was gently stirred for 18 hours at 5°C, and was then centrifuged at $18\ 000 \times g$ for 30 minutes at 5°C to yield a supernatant fluid which was used as the final antigen.

SERUM STANDARDS

An IgA positive reference serum pool was prepared from the sera of eight patients with acute acquired toxoplasmosis. All of the serum specimens had *Toxoplasma* specific IgM antibodies detected on both antibody capture ELISA and IFT, and *Toxoplasma* specific IgA antibodies detected on direct ELISA. In five patients a fourfold or higher increase in the IgG antitoxoplasma antibody titre was detected in consecutive serum samples on IFT. The other three patients had only one available serum sample with an IgG antitoxoplasma antibody titre greater than 2048 on IFT. These three patients had cervical lymphadenopathy of four months duration. The IgA reference serum pool was arbitrarily designated as having 1000 activity units per ml (U/ml). When the logarithm of absorbance values versus serum concentrations were plotted for individual serum samples (with varying activities) and the reference serum pool, the slopes of the curves were parallel, indicating that the reference serum pool was a suitable standard for determining the activity levels of the unknown serum samples.¹³ Serum standards, with values ranging from 10 to 1000 U/ml, were prepared by diluting the reference serum pool with a normal human serum (NHS) pool. The NHS pool was prepared by mixing equal parts of 10 serum samples from subjects with negative serology for *T gondii*.

DETERMINATION OF OPTIMAL REAGENT CONCENTRATIONS

The ELISA procedure was standardized using excess amounts of all reagents except the one being tested. The optimal concentration of the conjugate (affinity purified goat anti-

human IgA labelled with peroxidase) was based on conjugate titration experiments using human IgA coated polystyrene plates. For antigen titration, increasing amounts of *Toxoplasma* antigen (from 0.1 to 7.5 µg/ml) were assayed. The antigen titration was performed using 20 µl of the *Toxoplasma* positive serum pool per ml because previous serum titration studies showed that diluent serum concentrations greater than 15 µl serum/ml were necessary to achieve excess antibody.

ASSAY PROCEDURE

Toxoplasma antigen, diluted to 3 µg/ml in 0.1 M carbonate-bicarbonate buffer, pH 9.5, was used to sensitise the wells of flat bottomed ELISA plates (Corning, New York, USA). After sensitisation for one hour at room temperature and 14 hours at 4°C, the wells were washed three times with PBS containing 0.1% (v/v) Tween 20 and 200 µl of each serum sample (previously absorbed with Sepharose 4B-protein G), diluted 1 in 50 in PBS-Tween containing 1% bovine serum albumin, were added to the wells. After incubation for one and a half hours at room temperature and washing with PBS-Tween, as described previously, 200 µl of the optimal dilution of the conjugate in PBS-Tween were added to the wells. After conjugate incubation for one hour at room temperature and washing with PBS-Tween, 200 µl of the substrate system, containing 1.42 mM H₂O₂ (Aldrich, Wisconsin, USA) and 0.42 mM 3,3',5,5'-tetramethylbenzidine, were added to the wells. Fifteen minutes after the addition of the substrate, 50 µl 4N H₂SO₄ were added to each well to stop the colour reaction. The absorbance of each well was measured at 450 nm using a microtitre plate spectrophotometer. (Eti-System Reader, Sorin Biomedica, Italy). The final optical density for each well was determined by subtracting the mean optical density of three antigen controls on the corresponding plate. A standard curve for serum standards ranging from 5 to 1000 U/ml was included in each assay. All serum samples were tested in triplicate and the mean activity determined. From the mean activity, the appropriate standard curve was used to translate optical density readings into U/ml.

ASSAY PROCEDURE

Tachyzoites of the RH strain of *T gondii* were obtained as described previously. *T gondii* treated with formalin was obtained as described by Desmonts and Remington.¹⁴ Briefly, after washing the tachyzoites with PBS, the sedimented parasites were suspended in formalin diluted 1 in 5 in PBS. After 18 hours, the parasites were washed five times in PBS, suspended in an alkaline buffer (pH 8.7), containing 7.02 g NaCl, 3.09 g H₃BO₃, 24 ml 1N NaOH, 4 g bovine serum albumin, 1 g NaN₃, and enough distilled water to bring the volume to one litre. The suspension was then stored at 4°C.

The antibody capture agglutination assay was performed as described previously,^{3,10} with a few modifications. Briefly, each well on

a U-shaped polystyrene microtitre plate was coated with 100 µl of a 1 in 500 dilution of anti-human IgA monoclonal antibody (Biosoft, Compiègne, France) in PBS (pH 7.2). After blocking residual binding sites with bovine serum albumin, 100 µl serum dilutions ranging from 1 in 100 to 1 in 800 were added to the wells. The plates were then incubated for three hours at 37°C in a moist chamber. After washing, 100 µl of a tachyzoite suspension containing 1.5 × 10⁷ parasites/ml were added to the wells and the plates were then incubated for 16 hours at 37°C in a moist chamber. Each test included a negative, a positive, and an antigen control. The reaction results were determined using a mirrored plate reader. A complete tachyzoite carpet at the bottom of the well was recorded as positive and a sedimentation pattern forming a smooth button was recorded as negative.

Results

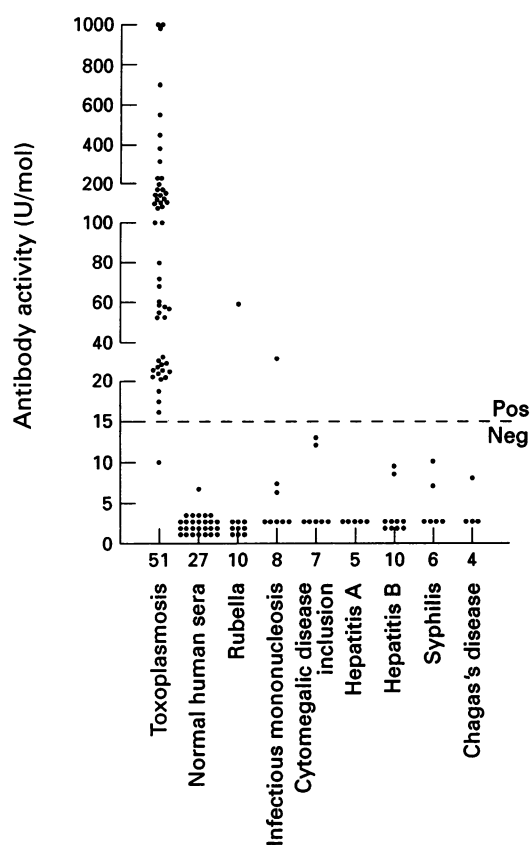
DIRECT ELISA

On the basis of antigen titration, an excess antigen concentration was achieved at concentrations greater than 2 µg/ml diluent. All assays requiring antigen excess were performed with 3 µg *Toxoplasma* antigen/ml. Conjugate excess was determined to be a dilution lower than 1 in 1500. All assays requiring conjugate excess were performed using a 1 in 750 conjugate dilution.

Each test included a standard curve covering a wide range of reactivity levels expressed as U/ml. Each serum sample was tested in triplicate and the mean activity was converted into U/ml using the standard curve. The results of all serum samples tested are shown in the figure. *T. gondii* positive samples had a range of activities from 10 to 1000 U/ml. All NHS samples had activities less than 7 U/ml. The J index¹⁵ was calculated at each level of reactivity from 10 to 20 U/ml. The formula for the index is as follows:

$$J = (a/b) + (c/d) - 1$$

where a is the number of subjects infected with positive serology, b is the total number in the infected group, c is the number uninfected with negative serology, and d is the total number in the uninfected group. The highest J index was obtained when 15 U/ml was used as the cut off value. At this cut off level, the sensitivity and specificity of the assay were 98 and 97%, respectively. Only one acute toxoplasmosis serum sample (10 U/ml) had a value below the cut off level. One infectious mononucleosis serum sample (31 U/ml) and one rubella serum sample (59 U/ml) had antibody levels above the cut off value. *Toxoplasma* specific IgM and IgG antibodies were not detected in the infections mononucleosis serum sample. The rubella serum sample was from a pregnant woman with clinical history compatible with rubella virus infection. The clinical diagnosis was confirmed by detection of rubella virus specific IgM antibodies on ELISA. In sequential serum samples from this patient *Toxoplasma* specific IgM antibodies were not detected on both ELISA



Results of the assay of 128 sera using a direct ELISA. The x axis shows the groups of patients and the number of serum specimens tested in each group. The y axis shows test results expressed as U/ml.

and IFT; a stable titre of 128 for *Toxoplasma* specific IgG antibodies was found on IFT.

Within-run and run-to-run variations were determined using a serum standard (250 U/ml). The mean coefficient of variation for 30 repeats of the assay to determine within-run variation was 4.9%. The run-to-run variation was determined from the assays of serum standards, in triplicate, on 11 alternate days. The mean coefficient of variation determined from the mean absorbance was 10.4%.

ANTIBODY CAPTURE ELISA

The antibody capture ELISA was performed using commercial kits from Sorin Biomedica, Italy. According to the manufacturer's instructions, most of the patients with acute toxoplasmosis have specific IgA levels higher than 40 AU/ml. Levels ranging from 10 to 40 AU/ml cannot be regarded as negative and their importance must be interpreted in association with specific IgM and IgG levels; levels below 10 AU/ml are regarded as negative.

Fifty one serum samples from patients with acute toxoplasmosis were assayed using the antibody capture ELISA. Only one sample had an *Toxoplasma* specific IgA antibody level below 40 AU/ml (35 AU/ml). All of the others had antibody levels higher than 40 AU/ml (54 to >160 AU/ml). One infectious mononucleosis serum sample had IgA levels greater than 10 AU/ml (24 AU/ml). In this sample *Toxoplasma* specific IgM and IgG antibodies

were not detected. All other serum samples from patients with heterologous infections and all those from normal subjects had IgA levels below 10 AU/ml.

ANTIBODY CAPTURE AGGLUTINATION TEST

All serum samples from patients with acute toxoplasmosis had *Toxoplasma* specific IgA antibody titres above 800. All serum samples from patients with heterologous infections and all those from normal subjects had antibody titres below 100.

Discussion

Most of infections caused by *T gondii* are asymptomatic and the minority of patients with clinical evidence of infection exhibit signs and symptoms that cannot be clearly attributable to the presence of the parasite.^{1,2} Thus, detection of *Toxoplasma* specific antibodies has been regarded as the most valuable tool for the diagnosis of toxoplasmosis. In this study we concentrated on the detection of *Toxoplasma* specific IgA antibodies in acute toxoplasmosis, using three immunological techniques. All three immunological techniques performed well with similar efficacy: 98% sensitivity and 97% specificity for the direct ELISA, 100% sensitivity and 99% specificity for the antibody capture ELISA, and 100% sensitivity and specificity for the antibody capture agglutination test. For the direct ELISA, preabsorption of sera with protein G enhanced both the sensitivity and specificity (data not shown). Despite this treatment, one false negative and two false positive reactions occurred. The false negative serum (10 U/ml) was positive on both the antibody capture ELISA (55 AU/ml) and the antibody capture agglutination test (antibody titre >800). The two false positive serum samples on direct ELISA, one infectious mononucleosis (31 U/ml) and one rubella serum sample (59 U/ml), were negative on both the antibody capture ELISA and the antibody capture agglutination test. One false positive reaction occurred on the antibody capture ELISA (an infectious mononucleosis serum sample (24 AU/ml)). This serum sample was negative on both the direct ELISA and the antibody capture agglutination test.

Preliminary studies showed that all serum samples from patients with heterologous infections had *Toxoplasma* specific IgA antibody titres below 100 on the antibody capture agglutination assay. On the other hand, sev-

eral samples from patients with acute *Toxoplasma* infection had relatively high titres (>3200); the titre of serial samples from some of these patients remained high for two months. Based on these experiments, we selected a range of dilutions from 1 in 100 to 1 in 800 for testing all serum samples. The antibody capture agglutination assay is easy to perform and the results from this report show that it may be used as a screening test for the diagnosis of acute toxoplasmosis in the routine laboratory.

Quantitative results were not directly comparable for *Toxoplasma* positive serum samples on both direct and antibody capture ELISA. This finding was not surprising as the intrinsic properties of the techniques and the antigen preparations used in the assays are different.

Our results regarding the detection of specific IgA antibodies in acute toxoplasmosis clearly show that detection of such antibodies is a useful diagnostic marker in the earliest phase of infection.

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