Standardized Quantitative Enzyme-Linked Immunoassay for Antibodies to *Toxoplasma gondii*

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The diagnosis of human toxoplasmosis has depended heavily on serological methods for detecting antibodies to *Toxoplasma gondii*. These principally include the classical Sabin-Feldman methylene blue dye test (MBD), the indirect immunofluorescence test (IIF), and the passive hemagglutination test (PHA). The current methods of choice for clinical purposes are the MBD and IIF tests (6). However, the former uses live organisms and a scarce "accessory factor," and IIF requires a fluorescent microscope and well-standardized fluorescein-labeled antibody preparations.

Recently, enzyme-linked immunosorbent assays (ELISA) have been applied to the detection of toxoplasma antibodies, particularly in microtiter plates (1, 2, 16–18). The microtiter ELISA is generally semiquantitative and may give erratic results due to the nonuniform absorbent quality of the plastic microtiter plates (3).

In our laboratories, an ELISA for detecting hepatitis B surface antigen in human blood samples has been developed which utilizes small plastic disks for covalent binding of the solid-phase reagents (6). This procedure was incorporated into a diagnostic kit (Cordia II, Cordis Laboratories, Miami, Fla.). As reported here, this format was modified for the quantitative determination of toxoplasma antibodies in human specimens. In addition, to establish a uniform basis of reference in the reporting of results, we measured the toxoplasma antibody levels in international units per milliliter, providing values directly traceable to the World Health Organization’s (WHO) first standard human antityoxoplasma serum preparation (19). In the present ELISA technique, serial dilution titrations and pretreatment of the test samples are not required; the total incubation time is short (1 h and 50 min); and either serum or plasma can be used. The communication describes several aspects of the general procedure and comparative results with MBD, IIF, and PHA assays.

**MATERIALS AND METHODS**

*T. gondii* organisms of the RH strain were harvested by differential centrifugation from peritoneal exudates of 3-day infected mice with heparin-containing saline (1 mg/ml). The suspensions were centrifuged and washed three times at 4°C (3,000 × g, 10 min). After being frozen at −20°C for at least overnight, they were thawed, sonicated, and centrifuged at high speed (45,000 × g, 30 min). The supernatant (soluble antigen) was collected and diluted with 0.1 M NaHCO₃, pH 9.6, to an appropriate concentration.

Special plastic disks containing surface isothiocyanate groups (13-mm diameter) were mixed with this antigen solution, agitated at 4°C overnight, washed, and lyophilized. These dried antigen-coated disks (containing 3 to 5 µg of protein each) were stored at 4°C in the presence of a desiccant until use. Antibodies to human immunoglobulins G and M were separately produced in goats, salted out with ammonium sulfate, and immunospecifically purified. They were then conjugated to calf alkaline phosphatase (PL Biochemicals, Milwaukee, Wis.) with glutaraldehyde (Polysciences Inc., Warrenton, Pa.) (5). The dialyzed antibody-enzyme conjugates were diluted to working concentrations in buffer at pH 8.0 (0.0025 M tris(hydroxymethyl)aminomethane [Tris], 0.2% bovine serum albumin, 0.001 M MgCl₂, 0.15 M NaCl, and 0.03 M sodium phosphate) containing 0.02% sodium azide. They were mixed proportionally for use and kept sterile at 2 to 8°C when not in use.
The human positive control was a pool of eight recalculated plasmas containing relatively high levels of *Toxoplasma* antibodies. It served as the secondary standard and was calibrated by the present ELISA method against the WHO's international standard for anti-toxoplasma serum, human, first standard, 1959 (19). The human negative control was a pool of recalculated human plasma with negligible amounts of toxoplasma antibodies (less than 20 IU/ml). Both controls were preserved with 0.1% sodium azide, made sterile by filtration, and stored in portions at 4°C until use.

For each test, one of the antigen-coated disks was incubated with 10 μl of sample diluted in 500 μl of specimen diluent (containing 0.01 M phosphate-buffered saline [pH 8.0], 6% bovine serum albumin, 0.05% Tween 20, and 0.1% sodium azide). After incubation at 37°C in a shaking water bath for 45 min, the sample solution was removed by aspiration, and the antigen disk (with antibody attached if present) was washed five times with 2.5 ml of 0.002 M 20 mM phosphate buffer (pH 7.2 to 8.0). Antibody-enzyme conjugate (0.5 ml) was then added to the disk, which was incubated as above for 45 min. The disk was again washed five times as above, transferred to an unused vial, and incubated as above for 20 min with 1 ml of the enzyme substrate, p-nitrophenyl phosphate (1 mg/ml in 0.028 M sodium bicarbonate buffer [pH 9.8], containing 0.01 M MgCl₂). The enzyme activity was stopped with 0.1 ml of 3 N NaOH and read with a spectrophotometer at a wavelength of 405 nm. To facilitate equipment used for the Cordis H procedure (Cordis Laboratories) was employed, i.e., devices for transferring and washing the disks.

The positive control and three fivefold serial dilutions of it in specimen diluent were tested simultaneously in each assay. The absorbance values and anti-toxoplasma activity (international units per milliliter) were plotted on log-log paper to establish a standard curve from which the anti-toxoplasma activity of each test sample was converted to international units per milliliter, based on its absorbance reading. Tests with the WHO standard, as well as with the secondary positive control, revealed that the straight-line relationship between absorbance readings and the anti-toxoplasma activity covered a range from 2 to 1,000 IU/ml. Because of the linearity of the relationship, the conversion from absorbance value to international units per milliliter could be made easily and rapidly with minimum error with an inexpensive hand-held calculator (e.g., Texas Instruments TI-55).

In addition to blood samples from local volunteers or blood donors, we obtained serum samples from three laboratories doing routine toxoplasma antibody immunoassays for clinical purposes and assayed them in a coded manner (after they had been tested in the laboratories supplying them) by the PHA, MBD, or IIF procedures. Comparisons of titers obtained by the present ELISA test and these other tests were analyzed by the correlation coefficient of Pearson and Lee (R) (10) after logarithmic transformation (15), with the negative results assuming the titers to be either 1, or one or more dilution level(s) below the lowest level determined in the titration system (15). The nonparametric rank correlation coefficient of Spearman (Rₜ) (10) was also used to avoid the bias due to this assumption for negative results, especially where they covered a wide range of titers (e.g., less than 16). For the same reason, where the sample size was large enough for a meaningful conclusion, results from samples with titers of 16 or greater were also separately analyzed by R. In addition to the conventional arithmetic calculation for standard deviation and coefficient of variation, the geometric standard deviation and geometric mean were also used for comparison of reproducibility of the present ELISA with other test results, as recommended by the Center for Disease Control (15).

**RESULTS**

A typical standard curve established with serial fivefold dilutions of the positive control is shown in Fig. 1. It can be seen that a straight-line relationship was obtained on a log-log scale and that the coefficient of correlation of the international units per milliliter and absorbance values in the example shown was 0.998. This coefficient was generally greater than 0.995. Similar straight-line relationships were found with the WHO primary standard, as well as with several individual high-titered serum specimens. The slopes of these lines tended to be similar.

Numerous parameters in the assay were varied singly to study their effects on the final absorbance values, as well as on the linearity and slopes of the standard curves. The variables included such factors as the amount of antigen used for coating the disks, incubation times, volumes of test sample, volumes of conjugates, etc. The results are summarized in Fig. 2. When various amounts of antigen were used for coating, the absorbance achieved reached a plateau at 5 μg/disk. However, it was found that the absorbance values for the negative control and background decreased when the disk was coated with more antigen, resulting in greater slopes for

![Fig. 1. Example of a typical standard curve of standardized quantitative ELISA for toxoplasma antibodies. Experimental data indicate that this straight-line relationship covers a range at least from 2 to 1,000 IU/ml.](http://jcm.asm.org/Downloaded_from)
the standard curves. When the concentration of substrate was increased, the slope dropped appreciably, although the absorbance increased slightly. Both absorbance and slope decreased substantially as the volume of substrate (at 1 mg/ml) was increased. Increased volumes of test sample increased the absorbance values slightly but decreased the linearity and slope of the standard curve. Varying the incubation times with the test sample, or with the conjugate, had relatively little effect on these values, whereas increasing the substrate incubation time increased the absorbance, but had only a minor effect on the slope. Increasing the conjugate volume or its concentration tended to increase the absorbance and the slope, but also increased the background absorbance unacceptably. Background absorbance decreased to a steady level as the concentration of bovine serum albumin in the specimen diluent was increased to 4% and above. Incubation at 37°C gave increased absorbance and provided better standard curves than at room temperature. In general, these data indicate that many of the conditions for the assay are not critical and could be tailored to meet particular needs within most of the parameters studied.

Assays of specimens derived from a single bleeding from the same donors indicated that either serum or plasma could be used in the ELISA assay, with equivalent results. Fifteen volunteers donated blood, and the anticoagulants used were heparin, citrate, dextrose citrate, and ethylenediaminetetraacetic acid. Five of the donors showed toxoplasma antibodies 200 IU/ml or higher, and three showed values of 10 IU/ml or less. After correction for any dilution factor due to the anticoagulant used, it was found that the toxoplasma antibody levels in the serum and various plasmas of any individual determined by ELISA were equivalent, i.e., they were within the assay error (average coefficient of variation, 15%).

The reproducibility of the ELISA procedure was quite satisfactory, as shown in Table 1. The "within-run" reproducibility of the assay showed

![Graph showing effect of some parameters on absorbance values of positive control and regression coefficient (slope) of standard curves.](image)

**TABLE 1. Within-run reproducibility of ELISA for Toxoplasma antibodies**

<table>
<thead>
<tr>
<th>Test specimens (no. of replicates)</th>
<th>Absorbance</th>
<th>IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arithmetic mean (% C. V.)*</td>
<td>Geometric mean X/+ SD*</td>
</tr>
<tr>
<td>A (9)</td>
<td>0.42 (9.2)</td>
<td>0.42 X/+ 1.10</td>
</tr>
<tr>
<td>B (9)</td>
<td>2.80 (6.0)</td>
<td>2.80 X/+ 1.06</td>
</tr>
<tr>
<td>C (9)</td>
<td>1.58 (5.2)</td>
<td>1.58 X/+ 1.05</td>
</tr>
<tr>
<td>D (8)</td>
<td>0.14 (3.3)</td>
<td>0.14 X/+ 1.03</td>
</tr>
<tr>
<td>E (8)</td>
<td>0.84 (5.0)</td>
<td>0.84 X/+ 1.05</td>
</tr>
<tr>
<td>F (8)</td>
<td>2.60 (7.0)</td>
<td>2.59 X/+ 1.07</td>
</tr>
<tr>
<td>G (8)</td>
<td>0.19 (3.0)</td>
<td>0.19 X/+ 1.03</td>
</tr>
<tr>
<td>Avg % C. V. or SD</td>
<td>5.5</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* C. V., Coefficient of variation.
* SD, Standard deviation.
an average coefficient of variation of 5.5% and a geometric standard deviation of 1.06 in the absorbance values. These values were 9.8% and 1.11, respectively, after conversion to international units per milliliter. Tests of day-to-day interlaboratory reproducibility showed approximately equivalent results.

The specificity of the ELISA values for toxoplasma antibodies was confirmed by absorption experiments. In the examples shown in Fig. 3, six human serum specimens (5 positive and 1 negative) were preincubated with T. gondii antigen, Entamoeba histolytica antigen, Trichi-nella spiralis antigen, or no antigen. The latter two parasitic antigens were crude extracts incorporated into diagnostic kits using immunodiffusion (Cordis Laboratories) and were known to possess relatively high specific antigenic activity, i.e., sufficient for gel diffusion techniques. The T. spiralis was derived from infected mouse tissue. The preincubated specimens were then assayed as above to determine whether neutralization of the antibody in the sample had occurred by the particular antigen in question. It is clear from the results that the toxoplasma antigen was the only preparation capable of significantly reducing the ELISA values of the positive specimens, indicating the immunological specificity of the present ELISA for toxoplasma antibodies. Reciprocally, when the disks were coated with E. histolytica antigens and used in similar neutralization experiments with human sera containing anti-amoeba antibodies, only E. histolytica antigen (not toxoplasma antigen) was shown to reduce significantly the anti-amoeba antibody activity (unpublished data).

Comparisons between the present ELISA and three other immunoassays (MBD, IIF, and PHA) are summarized in Fig. 4–7. High and significant correlation coefficients were observed in all instances. These data also indicated that specimens showing up to 30 IU/ml by ELISA were mostly negative by the other three assays. In addition, almost all specimens positive by the other assays showed greater than 10 IU/ml, and most of them were above 30 IU/ml. Four of the 206 specimens shown in Fig. 4 were nonreactive or reactive undiluted in the MBD test, but showed high ELISA values (>50 IU/ml). Absorption experiments as described above revealed that only the Toxoplasma antigen (but not normal mouse plasma, or amoeba or Trichinella antigens) was able to significantly reduce the high ELISA values in these specimens.

The Center for Disease Control (Atlanta, Ga.) distributes reference human serum controls for the assay of toxoplasma antibodies. One lot of these reference sera was assayed by ELISA, with good agreement in the results as shown in Table 2. These values are in accord with the distribution of values shown in Fig. 7.

**DISCUSSION**

The ELISA procedure described here has several advantages over other commonly used as-
with a single dilution of test sample. Moreover, the present assay was standardized against the WHO reference preparation, as the antibody concentrations were reported in international units per milliliter. Such a single basis of reference should minimize technical variations in different laboratories and permit more uniform measurements of toxoplasma antibodies in any laboratory using this procedure. This problem with the other assays is underscored by the description submitted with the WHO international standard for anti-toxoplasma serum. This single serum preparation was assayed in a number of qualified laboratories, and the titers found ranged from 400 to 10,000 in the MBD test, from 25 to 500 in the complement fixation test, and between 250 and 6,000 in the IIF test.

The ELISA described here was satisfactory for serum or plasma, and sodium azide as a preservative did not interfere with the results. No pretreatment of the test specimens is required, the assay is relatively rapid, and the reagents, which are ready for use, revealed considerable stability without any biohazard. Preliminary data indicated that all reagents could be kept refrigerated at 2 to 8°C for at least 2.0 years without noticeable effect on the test results. The antigen on the solid phase could even be kept at room temperature (23°C) for at least 10 months without loss of activity.
A rather good correlation was found between the ELISA values (international units per milliliter) and titers of toxoplasma antibodies found in several panels of test specimens assayed by MBD and IIF. In a separate, ongoing clinical research study with 34 specimens with MBD titers between 1,024 and 8,192, there was no indication of a plateau of the ELISA test results.
TABLE 2. ELISA assay results with Center for Disease Control toxoplasmosis control human sera

<table>
<thead>
<tr>
<th>Serum</th>
<th>Center for Disease Control-stated titer, IIF</th>
<th>ELISA observed (IU/ml)</th>
<th>Arithmetic mean ± SD</th>
<th>Geometric mean ×/× SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt;32</td>
<td>2.1 ± 0.3</td>
<td>2.1 ×/× 1.17</td>
<td></td>
</tr>
<tr>
<td>Low titer</td>
<td>32–128</td>
<td>62 ± 11</td>
<td>62 ×/× 1.19</td>
<td></td>
</tr>
<tr>
<td>High titer</td>
<td>512–2,048</td>
<td>203 ± 30</td>
<td>202 ×/× 1.17</td>
<td></td>
</tr>
</tbody>
</table>

* Catalog no. KK0501, lot 76-0339K.
* Measured in six experiments at different times.
* SD, standard deviation.

with these high-titered samples (unpublished data). Failure to achieve even higher correlations may be due to two factors. The MBD and IIF procedures allow a plus-or-minus fourfold error in the endpoint titers (12). Thus, a sample with a “true titer” of 1:94 may reveal titers of 1:16 or 1:256 in replicate assays. The reproducibility of the ELISA is considerably better, showing a geometric standard deviation of 1.11 in international units per milliliter, which is smaller than most of those reported for immunoglobulin quantitation (13, 14). The latter, in turn, has been found to provide appreciably better reproducibility than those serological tests using serial titrations, which allow accuracy to within one twofold dilution (14). In addition, the ELISA determination yields results which are presumably a summation of all the antibodies directed against the several toxoplasma antigens (at least seven) known to induce antibody responses in humans (4, 7, 9, 11; unpublished data, G.R.O.), since the preparations used to coat the disks were total extracts of the organisms prepared by very mild sonicadion procedures. In contrast, the MBD and the IIF tests undoubtedly measure a more limited set of antibody responses, presumably those situated on the surface membrane of the toxoplasma organisms (9).

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LITERATURE CITED


