Enzyme-linked immunosorbent assay for quantitation of toxoplasma antibodies in human sera

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SUMMARY An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of toxoplasma antibodies using a single serum dilution (1:800) in conjunction with a standard curve. Antigen was prepared from Toxoplasma gondii cultivated in human cell cultures. A nearly linear relationship was found between the logarithms of the absorbance values of 120 human sera at a dilution of 1/800 and the titres as determined by an endpoint dilution ELISA. The reproducibility of the single dilution ELISA was excellent; the coefficients of variation for within-day and day-to-day tests were less than 15%. A close correlation was found between the results obtained with ELISA, indirect immunofluorescence (IF), and complement fixation (CF). The titres in ELISA were 20 to 40 times higher than in IF and 200 to 1000 times higher than in CF.

Since its introduction by Engvall and Perlmann1 and van Weemen and Schuurs2 the enzyme-linked immunosorbent assay (ELISA) has gained wide application for detecting antibodies to various infectious agents. In the last few years, ELISA has been developed for application in toxoplasmosis. Variable results have been obtained in comparative studies with ELISA and established techniques for measuring antibodies to Toxoplasma gondii.3–7 In addition to the many variables of the ELISA system itself, differences in antigen preparation may be responsible for variable results. It is evident that the test still needs to be standardised.

We report here an ELISA procedure which permits a precise and reproducible quantitation of toxoplasma antibodies by examination of a single serum dilution in conjunction with a standard curve using soluble antigen from parasites cultivated in human cell cultures. The sensitivity of the test was compared with that of immunofluorescence (IF) and complement fixation (CF).

Material and methods

ANTIGEN

Human larynx carcinoma cells (HEp-2) were grown in 200 ml Kimble flasks at 37°C in Eagle's minimum essential medium supplemented with 10% newborn calf serum. When a confluent monolayer was obtained, each flask was inoculated with 1 ml of a suspension containing 5 x 10⁶ toxoplasma parasites of a strain isolated in the The Netherlands (strain Deelen). The parasites were permitted to adsorb for 1 hour at 37°C. Subsequently, 9 ml Eagle's minimum essential medium containing 2% newborn calf serum were added. After incubation at 37°C for seven days, the supernatant containing parasites was harvested. After renewing the culture medium and further incubation at 37°C for three days, another harvest was possible. At each harvest parasites were collected from the supernatant by centrifugation at 1000 g for 10 minutes, followed by three washings of the pellet with 0.1 M phosphate buffered saline (PBS) at pH 7.2. The average yield was 100 x 10⁶ to 130 x 10⁶ parasites per flask. The pellet was resuspended in PBS and, after three cycles of freeze-thawing, subjected to centrifugation at 3000 g for 15 minutes. The supernatant was stored at −70°C until used. The protein content of the antigen suspension as determined by the Lowry method was approximately 0.61 mg/ml. The optimal antigen dilution in ELISA was determined by checker board titration. An antigen dilution of 1/100 gave the highest ratio between positive and negative control samples, and this was used in all tests.

SERUM SAMPLES

Sera were obtained from patients with symptoms suggestive of toxoplasmosis. The sera were stored at −20°C for several weeks before being tested.
ELISA PROCEDURE
The test was performed essentially as described by Voller et al. A 0-2 ml amount of the 1 in 100 diluted antigen suspension was added to the wells of microhaemagglutination plates (Cooke Microtiter M29AR). After overnight incubation at 4°C the plates were washed three times in PBS containing 0-05% Tween 20. Then serial twofold dilutions of serum were made in the antigen-coated plates using PBS-Tween supplemented with 2% fetal calf serum (FCS). The starting serum dilution was 1/100, and the final volume of diluted serum in each well was 0.1 ml. In the single dilution ELISA 0.1 ml of 1 in 800 diluted serum was added to each of two antigen-coated wells. After incubation at 37°C for 2 hours in a humidified atmosphere the plates were washed three times with PBS-Tween, and a 0.1 ml amount of 1 in 1000 diluted conjugate was added to each well. After 1 hour's incubation at 37°C in a humidified atmosphere the plates were again washed three times with PBS-Tween, and 0.1 ml of substrate solution was added. The substrate solution containing o-phenylenediamine (Sigma) in 0.1 M PBS at pH 6.0 (1 mg/ml) and 0.1% of a 30% solution of H₂O₂ was prepared immediately before use. After 30 minutes' incubation in the dark at room temperature the reaction was terminated by adding 0.1 ml of 4N H₂SO₄. The colour intensity indicative of the antibody concentration in the serum was determined by spectrophotometry (Vitatron DCP); the results were expressed as the absorbance at 492 nm. In the single dilution ELISA the mean value of two tests was calculated.

END POINT DILUTION ELISA
There is as yet no generally accepted formula for determining antibody titres by ELISA. An antibody titre may be defined as the highest dilution at which the absorbance value of a serum differs significantly from those of negative sera. 'Significantly different' may be defined as three standard deviations above the mean OD value of the population of negative sera. However, a procedure for determining antibody titres based on such a definition is not practical since a large series of negative sera in each plate need to be tested, thus limiting examination of patients' sera. To circumvent this limitation, we applied the following procedure. First, we determined the mean absorbance value and the standard deviation of 44 sera diluted 1 in 100 which were negative in IF. In the same test the absorbance value of a 1 in 100 diluted negative control serum was determined. The latter serum was a pool composed of a number of negative sera. The absorbance value corresponding to three standard deviations appeared to be equal to 40% of the OD value of the negative control serum. In all subsequent tests 40% of the OD value of the negative control serum was used as a measure of a significant difference between a patient’s serum and negative sera.

To determine antibody titres by end point dilution the difference was calculated between the absorbance value of each dilution of a patient’s serum and that of the same dilution of the negative control serum examined in the same plate. Titres were expressed as the serum dilution at which this difference reached a value equal to 40% of the absorbance value of the 1 in 100 diluted negative control serum. The end point dilution was calculated by interpolation with readings at succeeding serum dilutions.

SINGLE DILUTION ELISA
To determine antibody titres by single dilution ELISA the absorbance value of a 1 in 800 diluted patient’s serum was compared with the same absorbance value and the corresponding titre of a standard curve. As will be described in the Results section, serum pools with varying titres as determined by end point dilution were used to construct the standard curve.

CONJUGATE
A commercial peroxidase-conjugated antihuman immunoglobulin G (γ chain specific, Institut Pasteur, Paris) was used. The conjugate was diluted in PBS containing 2% FCS and 0.05% Tween 20. The optimal dilution as determined by checker board titration was 1/1000, and this was used in all tests.

IMMUNOFLOUORESCENCE
The procedure was that described previously by us. Cryostat sections of brain tissue from mice infected intracerebrally three days previously with the Deelen strain of toxoplasma were allowed to react with a patient's serum, washed, and then allowed to react with a polyclonal fluorescein-conjugated antihuman antiserum (Progressive Laboratories, Inc) at a dilution of 1/40.

COMPLEMENT FIXATION
Antigen was prepared from parasites harvested from the peritoneal cavity of mice infected three days previously. Subsequent treatment was the same as that for preparation of the ELISA antigen. The CF procedure was the microtitre technique described by Casey.

Results
COMPARISON OF SINGLE AND END POINT DILUTION TESTS
A dose response test was performed in serial twofold
ELISA for toxoplasma antibody quantitation

Dilutions on five different positive human sera and on the negative control serum. The absorbance values, after subtracting the OD value of the negative control serum in the corresponding dilution, were plotted logarithmically against serum dilutions. Linear curves were obtained between 0.06 and 3.0 absorbance units in all positive sera (Fig. 1). Outside this range levelling of the curve was found similar to the findings with ELISA for influenza A antibodies described by Leinikki and Pässilä. The slopes of the curves for the five sera were nearly the same. Furthermore, a linear curve was obtained when antibody titres of these sera as determined by end point dilution ELISA were plotted against their absorbance value at a dilution of 1/800 (insert of Fig. 1). This suggests that the use of a standard curve is permissible.

Essentially similar results were observed in experiments with 83 other sera with varying antibody titres. The slopes of the linear portions of the dose response curves were calculated (Table 1). Only in high-titred sera were slightly lower values obtained, indicating that the slopes of these sera were less steep. The mean of the correlation coefficients of regression lines of the individual sera exceeded 0.99 in each group.

To determine whether ELISA with only one serum dilution would give a reliable estimate of the antibody level, 120 patients’ sera were examined by testing a single serum dilution (1/800) as well as by the end point dilution ELISA. The absorbance values at the 1/800 dilution were plotted against the titres determined by end point dilution (Fig. 2). Ideally, the points in Fig. 2 should lie on a straight line. In our experiment, we obtained by linear regression analysis a satisfactory correlation coefficient ($r^2 = 0.91$).

Next we made serum pools with varying antibody titres as determined by end point dilution ELISA; each pool consisted of a number of sera with approximately the same titre. The absorbance values of these serum pools at a dilution of 1/800 were compared with their antibody titre as determined by the end point dilution test similar to the experiment depicted in the insert of Figure 1. Again a straight line was obtained as calculated by linear regression analysis ($r^2 > 0.98$). In all subsequent single dilution tests, the serum pools were tested in the same way to construct a standard curve. The titre of an individual serum was obtained by comparing the absorbance value of the 1/800 dilution of this serum with the same absorbance value and the corresponding titre of the standard curve.

**Table 1** Slopes of dose response curves for 83 sera in end point dilution ELISA

<table>
<thead>
<tr>
<th>Antibody titre</th>
<th>Number of sera</th>
<th>Slope* (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;2000</td>
<td>12</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>2000-10000</td>
<td>36</td>
<td>0.77 ± 0.05</td>
</tr>
<tr>
<td>10000-30000</td>
<td>16</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>&gt;30000</td>
<td>19</td>
<td>0.63 ± 0.08</td>
</tr>
<tr>
<td>All sera</td>
<td>83</td>
<td>0.72 ± 0.08</td>
</tr>
</tbody>
</table>

*The relation between absorbance value and serum dilution for the linear part of the dose response curve is expressed by the following equation: $\log$ (absorbance) = $C - b \log (1/\text{serum dilution})$ in which $C$ is a constant and $b$ is the slope of the curve.

**Fig. 2** Correlation between absorbance values of 120 sera at a dilution of 1/800 and antibody titres of these sera as determined by end point dilution ELISA.

Reproducibility of ELISA

To study reproducibility, we tested five replicates of each of seven sera on each of five different days by single dilution and end point dilution tests (Table 2). Coefficients of variation did not exceed 10% and
Table 2  Within-day and day-to-day reproducibility of ELISA

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Absorbance value</th>
<th>ELISA antibody titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within-day tests*</td>
<td>Day-to-day tests†</td>
</tr>
<tr>
<td>1</td>
<td>0.067 ± 0.007</td>
<td>0.075 ± 0.004</td>
</tr>
<tr>
<td>2</td>
<td>0.082 ± 0.006</td>
<td>0.082 ± 0.013</td>
</tr>
<tr>
<td>3</td>
<td>0.239 ± 0.037</td>
<td>0.250 ± 0.037</td>
</tr>
<tr>
<td>4</td>
<td>0.355 ± 0.027</td>
<td>0.391 ± 0.055</td>
</tr>
<tr>
<td>5</td>
<td>0.550 ± 0.022</td>
<td>0.620 ± 0.074</td>
</tr>
<tr>
<td>6</td>
<td>0.797 ± 0.061</td>
<td>0.817 ± 0.088</td>
</tr>
<tr>
<td>7</td>
<td>1.90 ± 0.15</td>
<td>2.01 ± 0.24</td>
</tr>
</tbody>
</table>

*Five replicates of each serum were tested on the same day by single dilution ELISA. Mean absorbance ± standard deviation is given.
†Sera were tested on five different days by single dilution ELISA. Mean absorbance ± standard deviation is given.
‡The geometric mean titre ± 68% confidence interval is given. Antibody titres were determined on five different days.

15% in the within-day and day-to-day tests, respectively. Although the antibody titres in the single dilution test seemed to be slightly higher than those of the end point dilution test, the differences were not significant.

**Comparison with IF and CF**

Sera from 120 patients were assayed by IF, CF, and single dilution ELISA. ELISA proved as sensitive as IF for detecting toxoplasma antibody (Fig. 3) and, as might be expected, considerably more sensitive than CF (Fig. 4). The ELISA titres increased proportionally with the increase in the corresponding IF and CF titres; the correlation coefficients (rₐ) in Spearman's rank correlation test were, respectively, 0.91 (p < 0.001) and 0.93 (p < 0.001). The titres in ELISA were 20 to 40 times higher than in IF and 200 to 1000 times higher than in CF.

In addition, the WHO standard toxoplasmosis serum containing 500 international units per ml was examined by single dilution ELISA and IF in five separate tests. The serum was provided by Dr. Siim, of Copenhagen. The mean titre in ELISA was 45,000 (ranging from 38,000 to 53,000); the titre IF was 2048 in each of the tests.

**Discussion**

The advantages and problems of ELISA compared with those of various other serological tests have been extensively described by others and need not be repeated here. The end point dilution test used in this study enables one to compensate for test variabilities since cut-off values are related to the absorbance value of a 1 in 100 diluted negative control serum used in each day's test. The method requires some calculation, but this is easily done graphically or by the use of a calculator.

Another means of avoiding test variabilities is the
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use of a standard curve. The basic assumption underlying the use of a standard curve, not only in ELISA but in all comparative assays, is that the samples being compared are essentially identical and satisfy the condition of similarity, that is, their dose response relationship should have the same pattern. We showed that it was possible to construct a standard curve using serum pools with varying antibody titres. To obtain the ELISA antibody titre of an individual patient’s serum the absorbance value of a serum dilution of 1/800 was compared with the same value of the standard curve. We chose this high dilution because in this way absorbance values within the linear part of the dose response curves were obtained for most sera. The use of a single serum dilution in conjunction with a standard curve permitted a precise and reliable determination of antibody titres. Because of the high dilution used, the method lends itself very well to the determination of antibody titres in very small quantities of serum or plasma, for instance, in heel- or finger-prick blood droplets from infants. Other investigators have also reported the use of a standard curve obtained from serial dilutions of a reference serum for the assay of antibodies to rubella virus and to foot and mouth disease virus.

Reproducibility of the ELISA for toxoplasma antibodies was not affected by the large degree of inconstant antigen adsorption as described by Chessum and Denmark. However, in some batches of microtitre plates, ‘edge phenomena’ were observed, indicating the need to test each batch of microtitre plates for its suitability as solid phase in ELISA tests. The reason for this ‘edge phenomenon’ is not clear. It was not caused by test conditions but is possibly due to the manufacturing process of the plates.

In our test procedure, ELISA corresponded very well with IF in a range of approximately eight twofold serum dilutions from 1/64 up to 1/8000. In sera with IF antibody titres of 1/16000 or higher and 1/32 or lower, the correlation was less satisfying. Previous authors have reported that the use of a single dilution was not adequate for quantitative tests. The discrepancy with our findings is possibly due to the fact that these investigators used percentage transmittance as a predictor of the end point dilution factor and did not compare the results with the values of a standard curve.

Although ELISA is perhaps essentially a simple test to perform, more studies have to be done to standardize the test and to compare it with existing serological techniques under day-to-day operating conditions before the method can become an accepted serological procedure in routine diagnosis. We are indebted to Ria Heeren and Tini Tangelder for their excellent technical assistance. We thank Marij Gielen and Dr FWA Heessen for preparing the antigen.

References


Requests for reprints to: Professor J van der Veen, Department of Medical Microbiology, Geert Grooteplein Zuid 24, 6500 HB Nijmegen, The Netherlands.
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