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An enzyme-linked immunosorbent assay with whole trophozoites of *Toxop/asma gondii* from serum-free tissue culture for detection of specific antibodies

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Abstract This paper describes a new procedure of preparation of the antigen for an enzyme-linked immunosorbent assay (ELISA) for detection of antibodies against *Toxoplasma gondii.* To examine the reliability of this ELISA using whole trophozoites produced in a serum-free tissue culture as an antigen, 221 sera were tested comparatively in the new system (TTE, total trophozoites ELISA), in the indirect fluorescent antibody test (IFAT), and in a commercially available ELISA using sonicated trophozoites as an antigen (STE,sonicated trophozoites ELISA). The ELISA with antigen lysate showed a good correlation with the IFAT; however, false-negative results were sometimes obtained. The TTE was performed with all sera in two modifications: one test with an anti-IgG conjugate (G-TTE) and the other with an anti-Ig-G, -M, -A conjugate (GMA-TTE). In none of these TTE modifications were insensitivities observed; however, the G-TTE seems to offer a clearer differentiation between specifically reactive and nonreactive findings. The present study shows that the ELISA with whole trophozoites produced in serum-free tissue culture might be used as an alternative test to the IFAT. This test combines the advantages of the ELISA system with the sensitivity and specificity of the IFAT.

Introduction

Numerous tests have been developed for serodiagnosis of *Toxoplasma* infections in man (Ho-Yen and Joss 1992). The dye test (SFT) and the indirect fluorescent antibody test (IFAT) are particularly widely used as they are based upon membrane antigen and thus enable detection of early antibodies. In the Austrian toxoplasmosis surveillance of pregnant women they are used as basic tests (Aspöck and Pollak 1992). Besides these established tests, some attempts have been made to fix almost pure membrane proteins (Lövgren et al. 1987) or whole trophozoites (Picher et al. 1982; Tomasi et al. 1986; Verhofstede et al. 1987) to microtiter plates for use as antigen in an enzyme-linked immunosorbent assay (ELISA), thus combining the advantages of an ELISA (automation, objective reading of results by a photometer) with those of SFT or IFAT (high sensitivity, high specificity). Verhofstede et al. (1987) came to the conclusion that correlation of the test systems mainly depends on the way of (chemical) treatment of the parasite cells, on the one hand, and on the type of binding procedure to the solid phase, on the other.

In many laboratories, *Toxoplasma* trophozoites are bred in the peritoneal cavity of mice. For several reasons [animal protection legislation and nonspecificities of mouse-derived antigens (Hermentin et al. 1987b)], most laboratories try to replace cultivation of *Toxoplasma* in animals by breeding in vitro. To avoid the incorporation of any serum component (fetal calf, chicken) in the antigenic structure of *Toxoplasma,* which can be observed in conventional tissue cultures (Hass] and Aspöck 1991), Hermentin et al. (1987a) and Hass] and Aspöck (1992) established a serum-free in vitro culture. This technique yields high quantities of almost pure trophozoites, albeit with some minor changes in the antigenic assembly (Hass] and Aspöck 1991).

Thus, we tried to set up a quick and reliable ELISA system for toxoplasmosis diagnosis in routine laboratory work, combining the objectivity and efficient performance of an ELISA with the sensitivity and specificity of the SFT or IFAT. This test should not be regarded as another adaptation of the ELISA system for detecting antibodies against *Toxoplasma;* its main advantage lies rather in an improvement of the cultivation and preparation of the antigen.

Our ELISA system uses, whole, formalin-fixed, serum-free in vitro cultivated *Toxoplasma* trophozoites as an antigen. It was compared with an IFAT, which is also based upon the use of whole and formalin fixed trophozoites as an antigen, and with a commercially available ELISA, which uses sonicated trophozoites as an antigen.

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Materials and methods

Indirect fluorescent antibody test

The IFAT was performed in a standardized manner according to the recommendations of the Bundesgesundheitsamt of Germany (Bundesgesundheitsamt 1976) using a goat anti-human Ig/fluorescein isothiocyanate (FITC) conjugate (Behringwerke AG, Marburg/Lahn, Germany). The *Toxoplasma* trophozoites (RH strain) were harvested from the peritoneal cavity of mice (OF 1, Swiss SPF) infected 2 days earlier and were washed three times by suspension in phosphate-buffered saline (PBS; 3 mM KH_2PO_4, 12 mill Na_2HPO_4, 0.116 M NaCl; pH 7.2) and centrifugation. For fixation the pellet was resuspended in formaldehyde-PBS (4%), washed again and restored in PBS to a concentration of $2x10^6$ trophozoites/ml. The suspension was examined for contamination with mouse cells, which had to be less than 0.01%.

ELISA with lysate obtained from sonicated trophozoites as antigen

For this test (STE) we used the test kit ETI-TOXOK-G of Sorin Biomedica (Saluggia, Italy) with an antigen obtained from sonicated and extracted *Toxoplasma* trophozoites (RH strain). The test was carried out according to the manufacturer's instructions. Sera were diluted 1:101 in sample diluent (PBS, 2% bovine serum albumin, 0.05% Tween 20). As an enzyme tracer, freeze-dried and peroxidase-conjugated goat anti-human lgG (Fe) diluted 1:100 in tracer diluent (PBS buffer, Tween 20, and preservatives) was used. The standard curve was calculated by four calibrators with a concentration of 15, 30, 75, and 150IU/ml, respectively (calibrated against the WHO reference serum of 1977), using PBS buffer as the zero point. The enzyme reaction was developed by a buffer solution of tetramethylbenzidine derivative and H_2O_2.

ELISAs with whole trophozoites as antigen

Toxoplasma antigen, trophozoites (RH-strain) were bred in serum-free in vitro culture (Hep-2 cells) as previously described (Hermentin et al. 1987a; Hassl and Aspöck 1992) and were fixed with formaldehyde-PBS (4%). The control serum of IFAT (Toxotrol>F<, bioMerieux, Marcy-L'Etoile, France) was used as the calibrator serum, diluted in sample diluent to five different concentrations (150, 50, 16, 5, and 2 units).

The ELISA was performed in microtiter plates (Costar, Cambridge, USA) using 100-p1 incubation volumes. After each incubation period, wells were rinsed four times with washing buffer (PBS, 0.05% Tween 20) using an automatic washer (Denley Wellprep, Denley, Billingshurst, UK). Each sample was examined twice. A pool of 69 sera that had proved to be seronegative in all available tests was used as a negative control. The optimal dilution of the antigen and conjugate was determined in preliminary experinvents by chessboard tritration.

The plates were coated overnight at 4° C with the antigen preparation at a concentration of 10' trophozoites/ml sodium hydrogen-carbonate (35 mM) sodium bicarbonate (15 mM) buffer (pH 9.6). Free binding sites on the surface of the wells were coated with sample diluent buffer for 30 min at 37° C. Plates were stored at 4° C until use. No loss of quality was found after a pre vious maximal storage of 4 months. The sera, negative control, and calibrators were diluted 1:101 in sample diluent, pipetted into the wells, and incubated for l h at 37° C. Affinity-purified, freeze dried, and peroxidase-conjugated goat anti-human lgG (Fc) used for the total trophozoite ELISA with an anti-IgG conjugate (G-TTE) and goat anti-human lg-G, -M, -A (light and heavy chains) used for the GMA-TTE (Jackson Immunoresearch, West Grove, USA) were restored with bidistilled water according to the manufactuer's instructions, diluted 1:1500 in sample diluent buffer, and added to each well. After another period of incubation for 30 min at 37° C, a solution of 9 parts chromogen [80 mg purified (Ellens and Gielkens 1980) 5-amino-2-hydroxybenzoic acid in 100 ml bidistilled water] and 1 part 0.05% H_2O_2 was added. After 20 min of incubation at room temperature in a dark environment, the absorbance was recorded at 405 nm (680 nm reference wavelength) on an ELISA reader (SLT Labinstruments, Grödig, Austria) and was converted using the software program SOFT 2000 (SLT Labinstruments) into antibody units based on lin/log regression of the results of the calibrators. If a serum sample produced higher absorbance than the highest calibrator, it was diluted 20-fold and retested and the results were multiplied by 20.

To demonstrate intraassay reproducibility, the coefficient of variance (CV) of the 2-fold measured optical density of the enzyme reaction from 84 randomly chosen sera was calculated.

Table 1 Titer ranges of the several groups of summarized ELISA results for comparison with IFAT

Group	G-TTE (U)	GMA-TTE (U)	STE (IU)
Negative	1–10	1–10	1–14
1	11–16	11–15	15–22
2	17–75	16–92	23–137
3	76–225	93–177	138–348
4	226–1193	178–636	349–1663
5	1194–2322	637–2804	1664–6140
6	>2322	>2804	>6140

Positive-negative borderline

The manufacturer of the STE declares 15 IU as the positive borderline. For the TTEs the ELISA results of IFAT-negative sera were examined on outliers and normal distribution. After removing outliers a cut-off value of mean plus threefold standard deviation was calculated as negative borderline.

Sera

According to their IFAT titers, 221 human sera from the pregnancy screening program were selected for testing in the STE and TTEs. For a comparison of all tests, the results of the ELISAs were formed into one negative and six positive groups. The titer ranges (expressed in international units or units) of these groups were selected in such a way that the number of sera per group were approximately the same as the number of sera per IFAT-titer group (Table 1).

Statistical analysis

The one sample Kolmogorov-Smirnov test was used to test the by pothesis that a sample would be of symmetrical normal-frequency distribution. Outliers were defined as values lying more than 1.5 interquartile ranges from the end of the quartiles. Correspondence between the test systems was computed using Spearman's correlation coefficient (r_h). For calculation of all statistics the program SPSS (SPSS for Windows: Professional Statistics Release 5.0; SPSS Inc., Chicago, USA) was used.

Results

Intraassay reproducibility

The coefficient of variance (CV) of the optical density of 84 randomly chosen sera ranged from 0 to 13%; 96% of the sera had a CV of less than 10%.

Table 2 Comparison of IFAT reciprocal titers), G-TTE, GMMA-TTE, and STE. The ELISA results are summarized in 7 groups. The boldface and italic typed numbers show the number of sera within $^{\pm 1}$ titer group deviation as compared with the titers obtained in IFAT. The boldface typed numbers show the number of sera with approximately the same titer equivalents in ELISA and IFAT (G G TTE, *GMA* GMA-TTE, *Neg.* negative, *Bord.* border line)

ELISA Titer[a] group	IFAT Neg.	Bord.	16	64	256	1000	4000	Sum
G	37	3						40
GMA Neg.	**36**	1						37
STE	**38**	2		1				41
G	2		2	1				5
GMA 1	3	2	2	1				8
STE		1	2		1			4
G	3	1	**31**	19	7			61
GMA 2	3	1	33	17	6			60
STE	4	2	**30**	19	5			60
G		1	22	***20***	*13*			56
GMA 3		2	16	22	*15*	1		*56*
STE		1	22	22	11	1		57
G		1	2	15	21	4		43
GMA 4			6	*15*	**19**	4		44
STE			3	10	23	7		43
G				2	2	4	2	10
GMA 5				1	*3*	3	2	9
STE				5	3	1	1	10
G						2	4	6
GMA 6				1		1	4	6
STE						1	5	6
Sum	42/42/42	6/6/6		57/57/57	57/57/57	43/43/43	10/9/10	6/6/6 221/220/221

[a] For an explanation of the titer ranges, see Materials and methods and Table 1

Calculation of the positive-negative cutoff value for TTE

After the removal of six outliers in both tests, a mean of 2.9 (4.8) units in the G-TTE (GMA-TTE) with a 1.3 (l .8)-unit standard deviation was calculated. Because of the insignificant differences between the results for evaluation, a cutoff value of 11 units was set for both tests.

Comparison of the tests

Correlation between the tests

The highest correspondence was observed between the G- and the GMA-TTE (r_s=0.93). The STE showed an r_{ti} value of 0.88 (0.87) as compared with G-TTE (GMA-TTE). IFAT correlates were similar for all ELISAs (G-TTE, r,=0.78; GMA-TTE, r_s*=0.77;* STE, rs 0.77).

Sensitivity

Five and six of the IFAT-negative sera, respectively, were found in the G- and GMA-TTE to have near-cutoff or low-positive values (titer groups 1 and 2), whereas in the STE, four sera with low-positive results (titer group 2) were found. Three of these sera were positive in all ELISAs, and two were positive only in the two TTEs. Two of the sera gave positive results only in the STE or GMA-TTE. Sera with borderline titers (1:±16) in the IFAT gave different results in the ELISAs, varying from negative to group 3 in the STE and GMA-TTE and from negative to group 4 in the G-TTE.

Using the IFAT, 173 sera were considered to be positive for *Toxoplasma.* All of them showed positive titers in the ELISAs, too, except for one serum with an IFAT titer of 1:64, which proved to be (probably false-) negative in the STE. A consecutive serum sample obtained from this person showed a positive titer in this test and a rising titer in all others. The sera of IFAT groups 1:16, 1:64, and 1:256 yielded wide and overlapping results within and between the groups in all three ELISAs, but only 9% (7%) of all IFAT-positive sera showed deviations of more than $^{\pm 1}$ step within the titer ranges obtained in the GMA-TTE and STE (G-TTE; Table 2).

Discussion

Bonhomme et al. (1994) could not find any change in immunoreactivity by modifications in the conformation of the major surface protein P30 (SAGI) of invasive tachyzoites. In contrast to these findings, Verhofstede et al. (1987) came to the conclusion that the preservation of the original structure of the trophozoites seems to be essential in serological tests. Thus, we tried to set up an ELISA system using whole, formalin-fixed trophozoites as an antigen. To avoid the incorporation of nonspecific components [mouse-derived antigens, serum components of the culture medium (Dard6 et al. 1990)], the trophozoites were bred in a serum-free in vitro culture. To examine the reliability of this ELISA using whole trophozoites cultivated in a serum-free medium as an antigen for detection of antibodies against *Toxoplasma gondii,* 221 human sera were comparatively tested in 4 assays.

The test preparation (coating procedure, storage of the coated plates, preparation of the reagents) proved to be uncomplicated and fit for implementation in routine diagnosis. Almost the total working process can be carried out automatically, leading to high accuracy and low costs. Carbonate buffer proved to be a suitable coating buffer for stable adhesion of the trophozoites to the surface of the microtiter wells; thus, storage over several months is possible without any loss of quality. The low coefficient of variance (CV) of the optical density between the 2-fold applied serum samples convincingly demonstrated the constant number of epitopes. The natural character of the surface epitopes of the membrane of *T. gondii* does not seem to be destroyed by interlinking fixation with formaldehyde. As no insensitivity was detected by using purified 5-amino-2-hydroxybenzoic acid to develop the enzyme reaction, this chromogen seems to be a practical exchange for the cancerogenic substances commonly used.

As compared with all other tests, the G-TTE was the only one that produced results that were always confirmed by at least one of the other tests. Another advantage of this assay is the relatively wide gap between negative and specifically positive reactions. In the GMA-TTE, unspecific background absorbance was measured, which sometimes makes a clear distinction between negative and positive results more difficult. Given the same treatment of the antigen and the application of exactly the same test procedure used in the G-TTE, the insufficient distinction must be the result of the conjugate, which has to be improved in further investigations. One serum sample gave a positive result only in the STE; another serum specimen, which was positive in the IFAT, GMA-TTE, and G-TTE, was negative in the STE. A similar experience has been made with this commercial kit in another study (Obwaller et al.1994), in which titer conversions of women, documented by consecutive serum samples, were detected later than in the IFAT.

A good correlation was found for all four tests in the positive titer range (Table 2). Sera with IFAT titers of 1:16 were rated higher in all ELISAs, whereas sera with high IFAT titers were assessed lower in the enzyme immunoassays. The numbers of results that might lead to different conclusions were correspondingly low in all ELISAs; however, the G-TTE corresponded best with the IFAT.

We could not find any disadvantage in the use of an antigen of trophozoites cultivated as previously described (Hermentin et al. 1987a; Hassl and Aspöck 1992). The high quality of this antigen consisting of almost pure parasites made it possible to produce an efficient and economic test. Serum free in vitro -cultivated *T. gondii* seem to be an advantageous replacement for conventionally in vivo- or in vitro-bred trophozoites. Furthermore, the results of this study lead to the conclusion that the anti-human IgG ELISA using whole, serum-free, in vitro-cultivated trophozoites as an antigen (G-TTE) can be considered a suitable assay for toxoplasmosis diagnosis, as it detects antibodies against membrane antigens quite reliably. Particularly when used in combination with a test for detection of IgM antibodies, it probably could not be surpassed by other test systems. Further studies should focus on an improvement of the conjugates such that the GMA-TTE might reach a similarly high quality as the G-TTE.

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