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DIAGNOSIS OF HUMAN TOXOPLASMOSIS BY A DOT ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY

A Dot enzyme-linked immunosorbent assay (Dot-ELISA) was standardized and evaluated for the serodiagnosis of human toxoplasmosis. Out of 538 serum samples tested by the immunofluorescence test for toxoplasmosis (IFAT-IgG) as reference test, 183 (34%) were positive at cut off 1:16 and 192 (36%) were positive for Dot-ELISA-IgG at cut-off 1:256. For Dot-ELISA, co-positivity was 0.94, co-negativity 0.94 and concordance 0.88 in relation to IFAT-IgG. These results suggest the usefulness of Dot-ELISA (cut-off titer of 1:256) for the serodiagnosis of human toxoplasmosis. The main advantage of this technique is simplicity, positive test can be visually identified (colored precipitate). It does not require a special equipment and it can be used as a qualitative test to screen large numbers of samples or as a quantitative assay to determine end-point titration of individual sera.

KEYWORDS: Human toxoplasmosis; Dot-ELISA; Serodiagnosis.

INTRODUCTION

During the past few years, there has been an increased interest in the serodiagnosis of parasitic diseases using techniques that are rapid, simple to perform and inexpensive. There are a number of reasons for the evolution of simpler and faster diagnostic assays for parasitic diseases from traditional procedures. Among these are increasing reagent costs and a need to perform tests by minimally trained individuals [16]. Traditional serologic procedures for diagnosis of human toxoplasmosis such as dye test [19], indirect hemagglutination [10], complement fixation [11] and indirect fluorescent tests [6] are tedious and difficult to standardize, reagent consumptive, require highly trained technicians and use expensive and electrically operated instruments such as fluorescent microscopes. In the last decade, the enzyme-linked immunosorbent assay (ELISA) has been developed to detect a large number of parasitic diseases [22]. This technique has the advantage of using small volumes of serum and antigen and a high sensitivity. However, this test has a number of drawbacks, including the need for trained personnel, requirement of photometers and the occurrence of aberrant readings due to plate-to-plate and well-to-well variation, the so-called "edge effect" [5, 8, 12]. In an effort to develop a simpler and less expensive immunoassay for toxoplasmosis detection, the ELISA format was modified. Nitrocellulose paper replaced plastic plates as the solid support for antigen binding and a preci-

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pitable chromogenic substrate was used which in the oxidized form appears as a colored dot on the nitrocellulose [9]. The Dot-ELISA has been widely accepted as a rapid and versatile test for the detection of parasitic diseases in humans and livestock as fascioliasis [24], cysticercosis [21] and goat toxoplasmosis [3]. Dot-ELISA for diagnosis of human toxoplasmosis was described [17]. The objective of this work is to standardize and evaluate the Dot-ELISA to detect anti-*T. gondii* antibodies for human diagnosis.

MATERIAL AND METHODS

Sera

A total of 548 human sera from 2 groups were utilized. Group 1 included 538 sera from the Toxoplasmosis Laboratory sera bank (ICB, UFMG). Group 2 included serum samples from 10 patients with positive results in a commercially available ELISA kit for *T. gondii* specific IgM (Toxonostika-IgM, Organon Teknika). Group 1 was evaluated by immunofluorescent (IFAT-IgG) and immunoenzimatic (Dot-ELISA-IgG). Group 2 was evaluated by IFAT-IgG, IFAT-IgM, Dot-ELISA-IgG, Dot-ELISA-IgM and antibody capture ELISA-IgM.

Antigen

In the tests tachyzoites (N strain) obtained from peritoneal fluid of infected mice were used as the antigen. The tachyzoites were separated from their host cells by centrifugation (at 300 g for 30 sec) and washed three times in PBS 0.15 M, pH 7.2 at 1600 g for 15 min. Part of the tachyzoites was used as antigen for IFAT (whole parasite), which was prepared by fixing an aliquot of tachyzoites in 5% formalin-PBS for 10 min at room temperature and washing three times in PBS as above. Then smears of organisms were prepared and air-dried on IFAT slides. The slides were stored at -20°C until used. The other part of tachyzoites was washed as above. Tachyzoites were disrupted by sonication using three periods of 1 min at 40 hertz, centrifuged at 13000 g for 30 min at 4°C and the supernatant was used as the crude soluble antigen for Dot-ELISA. Protein concentration was determined [14]. The soluble antigen (0.1µg of protein) was fixed in nitrocellulose discs of 5 mm diameter and blocked with 0.1 ml/well PBS 0.15 M, pH 7.2 containing 0.05% Tween 20 (PBS-T) for 15 min. The nitrocellulose discs with bound antigen were placed in the wells of microplates, which were sealed and stocked at -20°C.

Antibody tests

a) Indirect fluorescent antibody test (IFAT)

The IFAT (reference test) was performed as described previously [6] IFAT procedures and reagents were those routinely used in our Laboratory for the immunodiagnosis of toxoplasmosis. For use, IFAT slides were warmed to room temperature. Four-fold serial dilution, starting at a titer dilution 1:16 to 1:16000 were incubated on the slides in moisture chambers for 30 min. at 37°C. Fluorescein-conjugated anti-human IgG prepared in the Toxoplasmosis Laboratory, ICB, UFMG (f/p = 9) or anti-human IgM conjugate (Sigma) diluted according to titer in PBS with 0.02% Evans Blue were added to the slides. After incubation for 30 min at 37°C, the slides were washed, air-dried and viewed using a fluorescence microscope. Reactions were considered positive when the tachyzoites showed complete surface fluorescence.

b) Dot-ELISA

The test was executed as described previously [9], with modifications concerning the utilization of nitrocellulose discs and the amount of reagents [3]. For use, nitrocellulose discs in microplates were warmed to room temperature. The discs were incubated for 30 min with 100 µl of four-fold serial human serum dilutions (1:16 to 1:16000) in PBS-T washed 3 times in PBS-T and incubated for 30 min. with 100 µl of anti-human IgG (or anti-human IgM) conjugated with alkaline phosphatase (Sigma), diluted at 1:10.000 in PBS-T.

After washing as above, 100 µl of substrate solution (0.05 mg of 5-bromo-4-chloro-3-indolyl phosphate and 0.05 mg of nitro blue tetrazolium, Sigma, in 1.0 ml of Tris buffer pH 8.0) were incubated with each disc for 20 min. The reaction was stopped by the addition of distilled water. All steps were performed under agitation at room temperature. Reading was performed by direct visual observation of the discs. A well-defined purple precipitate corresponded to a positive result while absent when negative.

c) Antibody Capture ELISA-IgM

ELISA-IgM was performed as described previously [15] with modifications. For coating, microtiter plates were incubated with 0.1 ml/well (2µg/well) of goat immunoglobulins to human IgM (Sigma) for 18 h at 4°C. After washing 3 times with PBS-T, 100 µl of human sera at 1:64 were added in each well. After incubation for 1 h at 37°C and washing, the antigen extract (50 µg/ml) and conjugate (peroxidase-labeled

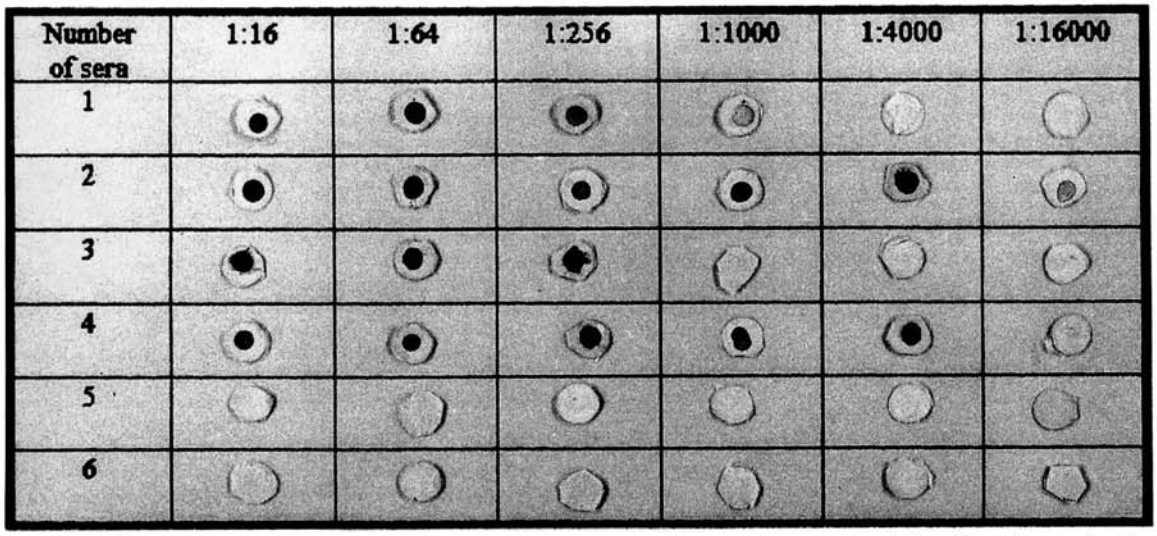

Fig. 1 - Dot-ELISA in nitrocellulose discs showing positive (1 to 4) and negative results (5 and 6). Positive results were indicated by a substrate-stained dot on nitrocellulose discs.

rabbit anti-*T. gondii* serum at dilution of 1:400) were added simultaneously. This conjugate was prepared in the Toxoplasmosis Laboratory, ICB, UFMG. After incubation for 1h at 37°C and washing, the substrate (o-phenylenediamine) was added and the microplates incubated for 20 min at 37°C. The reaction was stopped by the addition of 30 µl, 4 N sulphuric acid in each well. The absorbance at 490 nm was determined by using a microplate reader (BIO RAD model 3550).

Statistical Analysis

The statistical tests applied were: co-positivity and co-negativity indices [4]; concordance index [23], Mc Nemar's x^2 test[2] for comparison of proportions in two related samples and linear regression [20] to compare logarithm sera's titer of IFAT with Dot-ELISA.

RESULTS

Typical results of Dot-ELISA are shown in Fig. 1. The inclusion of positive and negative sera in each run ensured satisfactory performance of the test. Distribution of IgG antibody titers as evaluated by indirect fluorescent antibody test and Dot-ELISA for Group 1 are presented in Fig. 2. Out of 538 serum samples tested by IFAT, 183 (34%) were positive for toxoplasmosis at cut-off 1:16. While 248 (46%) were positive by Dot-ELISA at cut-off 1: 64, 182 (36%) were positive at cutt-off 1:256 and 169 (31%) were positive at cut-off 1:1000 (Table 1). A comparison of these two methods yields the following results: for a cut-off titer of 1:64, co-positivity (sensitivity) was 0.99, co-negativity (specificity) was 0.81 and Youden index

TABLE 1

Validate indices for **T. gondii** antibodies in human sera by immunofluorescence test (cut-off 1:16) and Dot-ELISA-IgG.

Dot-ELISA Cut-off	Co-positivity	Co-negativity	Youden	P-Dot-ELISA	P-RIFI	X^2
1:16*	0.99	0.63	0.62	0.58	0.34	122
1:64*	0.99	0.81	0.80	0.46	0.34	59.0
1:256	0.94	0.94	0.88	0.36	0.34	0.00
1:1000	0.84	0.96	0.80	0.31	0.34	3.67
1:4000*	0.56	0.99	0.55	0.20	0.34	32.7
1:16000*	0.21	1.00	0.21	0.07	0.34	143

* Significant difference between the proportion of positive sera in the two tests
P-Dot-ELISA = proportion of positive sera in Dot-ELISA
P-RIFI = proportion of positive sera in IFAT

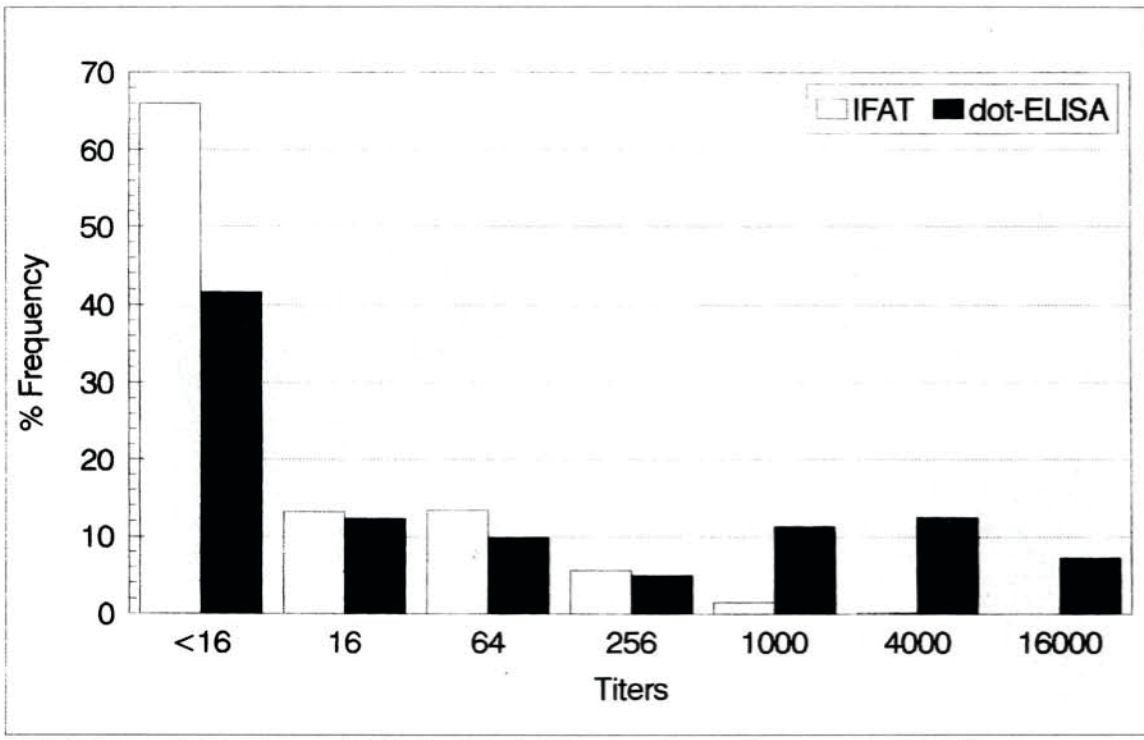

Fig. 2 - Distribution of anti-*Toxoplasma gondii* IgG antibody titers in 538 human sera by immunofluorescence test and Dot-ELISA.

(concordance) was 0.80. While the cutt-off was 1:256, co-positivity was 0.94, co-negativity was 0.94 and Youden 0.88. Using a cutt-off titer 1:1000, co-positivity was 0.84, co-negativity was 0.96 and Youden was 0.80. Indices obtained with cut-off values < 1:64 or ≥ 1:4000 were not satisfactory. Dot-ELISA serum IgG titer was correlated by linear regression analysis to IFAT IgG titers (Fig. 3). The coefficient between the two assays was 0.92.

Of the 10 sera investigated to detected IgM antibodies anti-*T. gondii*, only 5 gave positive results in IFAT-IgM while the 10 sera gave positive results by Dot-ELISA-IgM and by antibody capture ELISA-IgM (Table 2).

DISCUSSION

When analyzing the distribution of antibody titers

TABLE 2

Specific IgM antibody anti-*Toxoplasma gondii* in 10 human patients by antibody capture ELISA-IgM, IFAT (IgG and IgM) and Dot-ELISA (IgG and IgM)

Sera	ELISA-IgM	IFAT		Dot-ELISA	
		IgG	IgM	IgG	IgM
1	positive	1:4000	1:64	1:16000	1:4000
2	positive	1:256	1:64	1:4000	1:4000
3	positive	1:64	1:64	1:16000	1:1000
4	positive	1:8000	1:16	1:16000	1:1000
5	positive	1:1000	1:16	1:4000	1:4000
6	positive	1:256	<1:16	1:256	1:256
7	positive	1:256	<1:16	1:4000	1:1000
8	positive	1:256	<1:16	1:256	1:256
9	positive	1:256	<1:16	1:4000	1:256
10	positive	1:256	<1:16	1:16000	1:1000

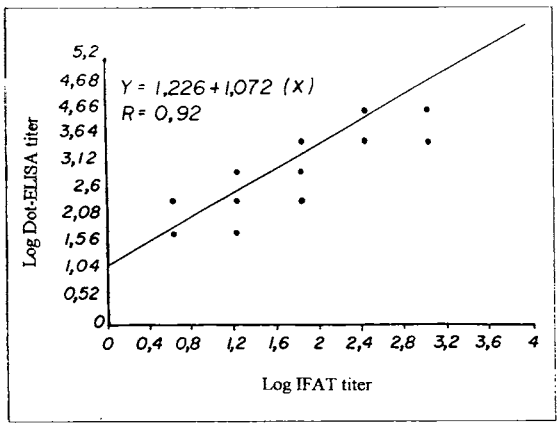

Fig. 3 - Correlation between IFAT and Dot-ELISA titers of 538 samples for anti-*T. gondii* antibodies. Each point represents more than one sample.

in the 183 positive samples for IFAT, it has been observed that in the Dot-ELISA these titers (95% of the samples) are, mostly, superior to those titers obtained in IFAT (Fig. 2). This phenomenon has been observed in the Dot-ELISA for serodiagnosis of human visceral leishmaniasis [16] and indicates that this assay regularly shows higher titers than other serologic tests. The reason for this phenomenon is mostly attributed to antigenic differences. Reacting antigens in IFAT consist mainly of membrane components, whereas in Dot-ELISA they composite both membrane and cytoplasmic components.

The results obtained suggest the usefulness of Dot-ELISA-IgG method (cut-off 1:256) for the serodiagnosis of human toxoplasmosis (high concordance with IFAT). Using cut-off 1:64 the test presented high sensitivity but low specificity and concordance. Cut-off 1:1000 presented high specificity but low sensitivity and concordance (Table 1).

Dot-ELISA-IgM was positive in all acute sera (Group 2) indicating high concordances with antibody capture ELISA-IgM. IFAT-IgM demonstrated low concordance with antibody capture ELISA-IgM (5 false negative results) probably by IgG competition [7]. The main advantages of Dot-ELISA are simplicity, positive test can be visually identified (colored precipitate), it does not require a special equipment and it can be used as a qualitative test to screen large number of samples or as a quantitative assay to determine endpoint titration of individual sera. The antigen fixed in the nitrocellulose discs stored at -20°C demonstrated stability when used in a period of 18 months after fixation.

The Dot-ELISA can be configured to suit a variety of diagnostic needs. When screening relatively large populations for toxoplasmosis prevalence at a single serum dilution, such as in epidemiological surveys, a nitrocellulose-based microtiter plate configuration would allow hundreds of samples to be tested with minimal use of reagent. When smaller numbers of specimens are tested, such as in small clinic, a dipstick [1, 18] or plastic card configuration [13] would be more suitable.

Besides the advantages mentioned, Dot-ELISA has some facilities in relation to the conventional technique which utilizes microtiter plates as support: (1) the nitrocellulose membrane (white) increases the contrast in the reading in relation to the microtiter plates making the discrimination of positive and negative reactions easier; (2) the test is performed at room temperature, and it is very fast; (3) a large number of samples can be tested simultaneously.

RESUMO

Diagnóstico da toxoplasmose humana através de um tese imunoenzimático Dot-ELISA

O Dot-ELISA foi padronizado e avaliado para o imunodiagnóstico da toxoplasmose humana. Entre 538 amostras testadas pela reação de Imunofluorescência Indireta (teste de referência) 183 foram positivas considerando-se os soros reagentes nas diluições ≥ 1:16 (34%). Utilizando a diluição 1:256 como título discriminante para o Dot-ELISA, foram observados 192 soros positivos (36%). A comparação entre as duas técnicas mostrou os seguintes resultados: co-positividade = 0,94, co-negatividade = 0,94 e youden = 0,88. Estes resultados mostram que o Dot-ELISA pode ser aplicado no imunodiagnóstico da toxoplasmose humana utilizando o título discriminante de 1:256. Entre as principais vantagens desta técnica podemos citar sua simplicidade, leitura visual sem uso de aparelho e utilização como teste qualitativo em levantamentos epidemiológicos ou como teste quantitativo para titulação de soros.

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