Rev. Inst. Med. trop. São Paulo
32 (5): 355-359, setembro-outubro, 1990

DOT-ELISA FOR DETECTION OF ANTI-*Cysticercus cellulosae* ANTIBODIES IN HUMAN CEREBROSPINAL FLUID USING A NEW SOLID-PHASE (RESIN-TREATED POLYESTER FABRICS). PRELIMINARY REPORT.

Adelaide José VAZ (1), Antonio Walter FERREIRA (2), (3), Mário E. CAMARGO (2), (3), Paulo Mutuko NAKAMURA (1) & Eide Dias CAMARGO (1)

SUMMARY

A dot enzyme-linked immunosorbent assay (DOT-ELISA) was developed to detect specific antibodies in cerebrospinal fluid (CSF) for human neurocysticercosis immunodiagnosis, with **Cysticercus cellulosae** antigen dotted on a new solid-phase. This was represented by sheets of a synthetic polyester fabric impregnated with a polymerized resin (N-methylol-acrylamide). A very stable preparation was thus obtained, the antigen being covalently bound by cross-linking with free N-methylol groups on the resin. Since robust, no special care was necessary for handling the solid-phase. The test could be performed at room-temperature.

From 30 CSF samples assayed, 14 were positive, from a group of 15 cases of neurocysticercosis, with titers from 1 to 128; 15 other samples, from normals or other neurological diseases, were all negative. Test characteristics seem to indicate it as adequate for epidemiological surveys. A more detailed study on sensitivity, specificity, reproducibility and the use in serum samples is being conducted.

KEY WORDS: Neurocysticercosis; DOT-ELISA; Polyester-resin; Solid-phase; Cerebrospinal fluid.

INTRODUCTION

Neurocysticercosis (NC) is the most important parasitic infection of the central nervous system (CNS) in undeveloped countries, and represents a serious public health problem due to its high prevalence and morbidity.

The heterogeneity of clinical signs and symptoms that occurs in NC difficults the diagnosis. So, clinical criteria, epidemiological data, X-rays, computerized axial tomography (CAT) or nuclear magnetic ressonance (NMR) brain scans, and immunodiagnostic tests are employed to elucidate the cases.

Several kinds of immunological tests with diversified degrees of efficiency have been used

(1) Instituto Adolfo Lutz — Divisão de Biologia Médica, Serviço de Microbiologia e Imunologia, Laboratório de Imunodiagnóstico. São Paulo, SP, Brasil.
(2) Instituto de Medicina Tropical de São Paulo — Departamento de Moléstias Infecciosas e Parasitárias, Laboratório de Soroepidemiologia. São Paulo, SP, Brasil.
(3) Biolab Diagnóstica S.A.
Address for correspondence: Dra. Adelaide José Vaz. Instituto Adolfo Lutz. Av. Dr. Arnaldo, 355. CEP 01246 São Paulo, SP, Brasil.

as an aid in the NC diagnosis. These include complement fixation, passive haemagglutination and indirect immunofluorescence tests[10, 12, 13, 14, 15, 16, 18, 21].

During the last decade immunoenzymatic assays have been employed on account of their high reliability. The enzyme-linked immunosorbent assay (ELISA) is useful for diagnostic purposes and more seldom for epidemiological surveys[7, 8, 11, 17, 21, 24, 25]. However, this test needs an ELISA-reader, not always available in field work or small laboratories.

Dot-immunobinding technique (DOT-ELISA) is more easily performed, precluding any special equipment, since results are directly observed. Also, this procedure allows the performance of a number of tests on a single assay strip, being thus useful in surveillance programs.

TÉLLEZ-GIRÓN et al., 1987, employed DOT-ELISA on nitrocellulose membranes to detect **Cysticercus cellulosae** antigens in cerebrospinal fluid (CSF) from neurocysticercosis patients, refering good results.

In this study, DOT-ELISA on a recently developed solid-phase support was used to detect the presence of anti-**Cysticercus cellulosae** antibodies in human CSF, in order to improve the NC immunodiagnostic possibilities. This support was made up of synthetic polyester fabric sheets coated with polymerized resin (N-methylol-acrylamide, NMA), thus containing free N-methylol groups able to form covalent bonds with functional groups (-OH and -NRH) of proteins and polyssaccharides present in the antigenic extract obtained from **Cysticercus cellulosae.**

MATERIAL AND METHODS

ANTIGEN — Cysticerci antigen extract preparation has been described in detail elsewhere[24]. Briefly, cysticerci were washed extensively and ground in a Potter glass homogenizer. The homogeneite was further disrupted by sonication, centrifuged at 6,500 g, and the supernatant fluid centrifuged at 9,000 g. This last supernatant was collected and stored at -20°C until use. Protein concentration of the antigenic extract was determined by the method of BRADFORD[4].

CSF SAMPLES — Fifteen samples came from patients with NC diagnosed by the usual criteria (clinical syndromes, positive CAT brain scans, positive CSF immunological tests, CSF biochemical and cytological consistent results); five CSF were from healthy individuals and ten CSF from patients with other neurological diseases (viral and bacterial meningites, cerebral tumor and intracranial hemorrhage).

DOT-ELISA PROCEDURE — Polyester fabric-resin sheets were prepared as described[3, 5, 20] and cut with scissors to an appropriate size, 8 x 12 cm. All steps for fixing antigen on the sheets were performed at room temperature (23-25°C), working solutions being made in triethanolamine-buffered saline solution (TBS) (29.25 g NaCl, 2.42 g $(CH_2CH_2OH)_3$, add distilled, deionized water and 1N HCl (about 20 ml) to pH 7.5, complete to 1 liter). Antigen in 1 μl amounts (0.5 μg protein) was dotted on the resin-treated fabric by aid of a microtitration plate as a pattern for the dots[2], and fixed by drying for 60 minutes at room temperature. Remaining free reactive sites were then blocked by soaking each sheet in about 30 ml of a 5% skim-milk (p/v) in TBS[9] in a bowl. This was shaken in a rotator at medium speed for two hours. After washing in TBS the sensitized and blocked sheets were kept at 4°C in tightly closed plastic bags until use.

For the use, 50 μl of CSF samples serially diluted in 1% skim-milk TBS were added to wells of a microtiter plate. The antigen sheet was adequately placed and fixed on the plate, which was turned in order to put samples in contact with respective antigen dots on the sheet[2]. After 60 minutes at room temperature, sheets were washed by shaking in three changes of TBS with 0.05% Nonidet-40 (NP-TBS), for ten minutes each. Horseradish peroxidase-labeled anti-human IgG (Sigma Chemical Co. St. Louis, USA) optimally diluted at 1:100 in 1% skim milk-TBS was added to wells of a microtiter plate, which was turned, as described, incubated for 60 minutes at room temperature and washed. Then, sheets were incubated for 10 minutes in the chromogen solution (prepared immediately before

use by adding 4 ml of a 3 mg/ml 4-chloro-1-naphtol solution to 20 ml of TBS containing 10 μl of 30% H_2O_2). Sheets were then washed three times in TBS and allowed to dry.

Positive results were indicated by the development of a well defined purple color on dots.

RESULTS

The dose-response curve for antigen in DOT-ELISA is shown in figure 1. The lowest antigen concentration giving the highest titer of 128 with positive CSF control was 0.5 μg/μl, or 0.5 μg of protein per dot. That amount was chosen as the working concentration. Concentrations above this one would be superfluous and concentrations of 0.25 μg or less led to faintly or no visible reaction in the 128 dilution of the positive control. Antigen, conjugate and diluent controls were always negative.

The optimal dilution of conjugate as determined by block titration was 1:100.

Results of a typical DOT-ELISA onto polyester fabric-resin are presented in figure 2. Positive reactions (coloured dots) are observed in rows number 2 (Titer = T = 4); 3 (T = 16); 4 (T = 8); 6 (T = 2) and 7 (T = 32), corresponding to CSF samples from NC patients. Negative reactions are observed in rows number 1 and 5 (CSF from healthy individuals). There was no color development in dots 1F, 1G and 1H, respectively,

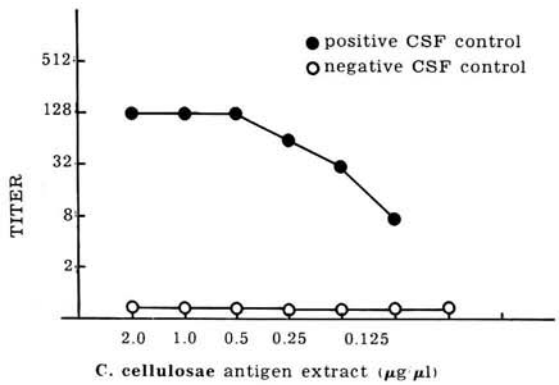

Fig. 1 — Determination of optimal antigen concentration for DOT-ELISA. Decreasing concentrations of antigen were dotted onto polyester fabric-NMA resin, incubated with positive and negative CSF controls and titers determined visually.

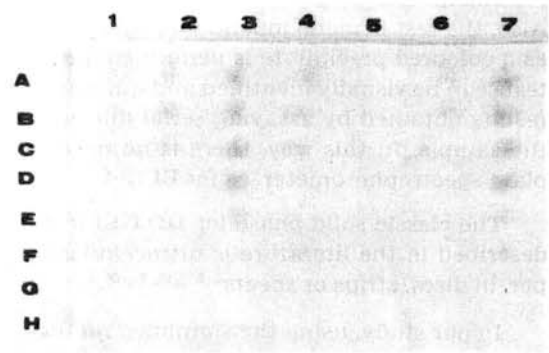

Fig. 2 — DOT-ELISA on polyester fabric-NMA resin using CSF (dilutions 1:1 to 1:128 from A to H) from patients with proven neurocysticercosis (rows 2, 3, 4, 6, 7) and healthy individuals (rows 1 and 5). CSF number 1 was diluted just to 1:16. Antigen, conjugate and chromogen-substrate controls are, respectively, in 1F, 1G and 1H positions.

antigen, conjugate and chromogen-substrate controls.

Table 1 shows the DOT-ELISA results for detection of anti-**Cysticercus cellulosae** antibodies in the 30 CSF samples studied, including 15 from patients with NC and 15 from the control group.

DISCUSSION

Enzyme immunoassays represent a truly powerful system in detecting or quantifying antibodies, antigens or haptens in biological fluids, by measuring the enzymatic activity as an amplifier of the reaction[6]. DOT-ELISA is a very sensitive, specific and reproducible test with a number of advantages over classical ELISA. In ELISA, concentration of antigen adsorbed per well is unknown and variable, with differences among brands and even lots of microplates as among wells in a same plate. In DOT-ELISA, a measu-

TABLE 1

Results of DOT-ELISA on polyester fabric-NMA resin for detecting anti-**Cysticercus cellulosae** antibodies in CSF samples from patients with NC (a) and control group (b).

		TITER							
Group	NR*	1	2	4	8	16	32	64	128	Total
a	1	2	2	1	—	5	3	—	1	15
b	15	—	—	—	—	—	—	—	—	15

* CSF non-reactive undiluted (1:1).

red amount of antigen is distributed in each dot. Also, the test is made much more simple since, as a coloured precipitate is performed, positive tests can be visually identified and quantitative results obtained by assaying serial dilutions of the sample. In this way, there is no need of a plate spectrophotometer, as for ELISA.

The classic solid-phase for DOT-ELISA, as described in the literature is nitrocellulose paper, in discs, strips or sheets[1, 2, 9,19, 22, 26].

In our study, using the simplified methodology, we introduce a new solid-phase for DOT-ELISA and started its evaluation. The solid-phase consists of a synthetic polyester fabric coated with crosslinked N-methylol-acrylamide resin. This support was assayed before in ELISA with good results[3, 5, 20, 24].

The DOT-ELISA in the support described was standardized for detection of anti-**Cysticercus cellulosae** antibodies in CSF from 15 patients with NC; all but one were positive with titers from 1 to 128 (geometric mean 9.8). All the CSF samples from a control group were negative even undiluted (Table 1). These results demonstrate the potencial value of the test in the immunodiagnosis of NC.

Since the binding of antigen to the solid-phase is covalent, so very stable, there is the possibility of a large routine use, as in field epidemiological surveys. In addition, all assay steps are performed at room temperature. A further advantage of the presently described solid-phase is the very low cost in relation to nitrocellulose sheets, as well as, the easy of handling in all steps of its use.

Further and more detailed studies mainly with respect to stability, reproducibility and cross reactions, and the use of serum samples, are being conducted and the results will be published later.

RESUMO

DOT-ELISA para pesquisa de anticorpos anti-Cysticercus cellulosae **em líquido cefalorraquiano empregando uma nova fase sólida (tecido de poliéster tratado com resina polimerizada). Dados preliminares.**

Foi desenvolvido o teste DOT-ELISA em tecido de poliéster tratado com resina polimerizada (NMA) para o imunodiagnóstico da neurocisticercose (NC) humana pesquisando anticorpos específicos em líquido cefalorraquiano (LCR). O teste pode ser empregado em levantamentos epidemiológicos e em laboratórios de média e baixa complexidade, já que todas as etapas são realizadas a temperatura ambiente, o antígeno é covalentemente ligado ao suporte permitindo obter um reagente estável, e o suporte sensibilizado não requer cuidados especiais no seu manuseio. Foram ensaiadas 30 amostras de LCR; todas as 15 amostras do grupo controle foram negativas e 14 (93,3%) das 15 amostras de LCR do grupo de pacientes com NC foram positivas (títulos de 1 a 128). Os estudos de sensibilidade, especificidade, reprodutibilidade, estabilidade e padronização do uso de soro como amostra estão em andamento.

ACKNOWLEDGEMENTS

We wish to thank Drs. José A. Livramento and Luis R. Machado of the Neurological Investigations Center, Faculdade de Medicina da Universidade de São Paulo, and Dr. Marcos V. da Silva, of Emílio Ribas Hospital, for providing CSF samples. We also thank Ms. Maria Emília G. Moura of Biolab Diagnóstica S.A. for preparing the resin-treated polyester fabrics.

REFERENCES

1. ARAÚJO, F. G. — A method for demonstration of antibodies to **Trypanosoma cruzi** by using antigen-coated nitrocellulose paper strips. **Amer. J. trop. Med. Hyg., 34:** 242-245, 1985.

2. BENNETT, F. C. & YEOMAN, L. C. — An improved procedure for the 'Dot Immunobinding' analysis of hybridoma supernatants. **J. immunol. Meth., 61:** 201-207, 1983.

3. BITTENCOURT, E.; GUIMARÃES, M.; CAMARGO, M. E.; FERREIRA, A. W. & MAKINO, M. M. — Immunoassays conducted with resin treated nylon fabrics. **J. Polymer. Sci.: Polymer Letters Edition, 21:** 717-722, 1983.

4. BRADFORD, M. M. — A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. **Analyt. Biochem., 72:** 248-254, 1976.

5. CAMARGO, M. E.; BITTENCOURT, E.; FERREIRA, A. W.; PERES, L. & CARVALHO, M. B. — Utilização de resinas sobre suportes inertes para fixação de covalente de componentes antigênicos ou anticorpos, como fase sólida em testes sorológicos. In: **JORNADA CIENTÍFICA DO**

INSTITUTO DE MEDICINA TROPICAL DE SÃO PAULO, 2, São Paulo, 1983. **Resumos.** p. 21.

6. CARLIER, Y.; BOUT, D. & CAPRON, A. — Enzymo-immuno assays. **Bull. Inst. Pasteur, 79:** 313-382, 1981.

7. COSTA, J. M.; FERREIRA, A. W.; MAKINO, M. M. & CAMARGO, M. E. — Spinal fluid immunoenzymatic assay (ELISA) for neurocysticercosis. **Rev. Inst. Med. trop. S. Paulo, 24:** 337-341, 1982.

8. COSTA, J. M.; MINEO, R.; LIVRAMENTO, J. A. & CAMARGO, M. E. — Detecção pelo teste imunoenzimático ELISA de anticorpos IgM anti-**Cysticercus cellulosae** no líquido cefalorraqueano na neurocisticercose. **Arq. Neuro-psiquiat. (S. Paulo), 43:** 22-28, 1985.

9. GUIMARÃES, M. C. S.; CELESTE, B. J. & FRANCO, E. L. — Evaluation of dot enzyme-linked immunosorbent assay for mucocutaneous leishmaniasis and comparison with microplate enzyme immunoassay. **J. clin. Microbiol., 24:** 364-367, 1986.

10. LANGE, O. — Síndrome liquórica da cisticercose encéfalo-meningéia. **Rev. Neurol. Psiquiat. (S. Paulo), 6:** 35-48, 1940.

11. LARRALDE, C.; LACLETTE, J. P.; OWEN, C. S.; MADRAZO, I.; SANDOVAL, M.; BOJALIL, R.; SCIUTTO, E.; CONTRERAS, L.; ARZATE, J.; DIAZ, M. L.; GOVEZENSKY, T.; MONTOYA, R. M. & GOODSAID, F. — Reliable serology of **Taenia solium** cysticercosis with antigens from cyst vesicular fluid: ELISA and hemagglutination tests. **Amer. J. trop. Med. Hyg., 35:** 965-973, 1986.

12. LIVRAMENTO, J. A. — **Contribuição de reações de imunofluorescência no líquido cefalorraqueano ao estudo da neurocisticercose.** São Paulo, 1980 (Tese de doutoramento — Faculdade de Medicina da Universidade de São Paulo).

13. MACHADO, A. J.; CAMARGO, M. E. & HOSHINO, S. — Reação de imunofluorescência para cisticercose com partículas de **Cysticercus cellulosae** fixadas a lâminas de microscopia. **Rev. Soc. bras. Med. trop., 7:** 181-183, 1973.

14. MARTINEZ-CAIRO, S.; RUIZ-MACIAS, C.; LÓPEZ-ROMAN, M. & MATEOS-GOMEZ, H. — Usefulness of concentrated CSF hemagglutination technic for the diagnosis of cerebral cysticercosis. **Arch. invest. méd. (Méx.), 11:** 347-359, 1980.

15. MOSES, A. — Dos métodos biológicos de diagnóstico nas cisticercoses. **Mem. Inst. Oswaldo Cruz, 3:** 320-327, 1911.

16. NASCIMENTO, E. & MAYRINK, W. — Avaliação de antígenos de **Cysticercus cellulosae** no imunodiagnóstico da cisticercose humana pela hemaglutinação indireta. **Rev.Inst. Med. trop. S. Paulo, 26:** 289-294, 1984.

17. NASCIMENTO, E.; TAVARES, C. A. & LOPES, J. D. — Immunodiagnosis of human cysticercosis (**Taenia solium**) with antigens purified by monoclonal antibodies. **J. clin. Microbiol., 25:** 1181-1185, 1987.

18. NIETO, D. — Cysticercosis of nervous system. Diagnosis by means of spinal fluid complement fixation test. **Neurology (Minneap.), 6:** 725-738, 1956.

19. PAPPAS, M. G.; HAJKOWSKI, R. & HOCKMEYER, W. T. — Dot-enzyme linked immunosorbent assay (DOT-ELISA): a micro technique for the rapid diagnosis of visceral leishmaniasis. **J. immunol. Meth., 64:** 205-214, 1983.

20. PERES, L. — **Desenvolvimento e caracterização de imunosorventes e avaliação de desempenho em ensaios imunoenzimáticos.** Campinas, 1986 (Dissertação de Mestrado — Faculdade de Engenharia Química da Universidade de Campinas).

21. PIALARISSI, C. S. M.; VAZ, A. J.; SOUZA, A. M. C.; NAKAMURA, P. M.; CAMARGO, E. D.; SILVA, M. V. & UEDA, M. — Estudo comparativo de testes sorológicos no diagnóstico imunológico da neurocisticercose. **Rev. Inst. Med. trop. S. Paulo, 29:** 367-373, 1987.

22. STERNBERG, J. & JEPPESEN, P. — Dot-blotting: a novel screening assay for antibodies in hybridoma cultures. **J. immunol. Meth., 64:** 39-43, 1983.

23. TÉLLEZ-GIRÓN, E.; RAMOS, M. C.; DUFOUR, L.; ALVAREZ, P. & MONTARTE, N. — Detection of **Cysticercus cellulosae** antigens in cerebrospinal fluid by dot enzyme-linked immunosorbent assay (DOT-ELISA) and standard ELISA. **Amer. J. trop. Med. Hyg., 37:** 169-173,1987.

24. VAZ, A. J. & FERREIRA, A. W. — Imunodiagnóstico da neurocisticercose: teste imunoenzimático com antígenos quimicamente ligados a suportes para pesquisa de anticorpos em soro e líquido cefalorraquiano. **Rev. Inst. Med. trop. S. Paulo, 30:** 1-10, 1988.

25. VIANNA, L. G.; MACÊDO, V.; COSTA, J. M.; MELLO, P. & SOUZA, D. — Estudo soroepidemiológico da cisticercose humana em Brasília, Distrito Federal. **Rev. Soc. bras. Med. trop., 19:** 149-156, 1986.

26. ZHENG, G. Y.; ZHAO, R. L. & FENG, X. H. — Dot immunobinding assay in the serodiagnosis of human hydatid disease. **Amer. J. trop. Med. Hyg., 35:** 812-814, 1986.

Recebido para publicação em 13/11/1989.