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DOT-ELISA FOR THE DETECTION OF ANTI-*Cysticercus cellulosae* ANTIBODIES IN CEREBROSPINAL FLUID USING A NEW SOLID PHASE (RESIN-TREATED POLYESTER FABRIC) AND *Cysticercus longicollis* ANTIGENS

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SUMMARY

A dot-ELISA was developed for the detection of antibodies in CSF in the immunologic diagnosis of human neurocysticercosis, using antigen extracts of the membrane and scolex of *Cysticercus cellulosae* (M+S-Cc) and, alternately, membrane (M) and vesicular fluid (VF) of *Cysticercus longicollis* (Cl) covalently bound to a new solid phase consisting of polyester fabric treated with N-methylol-acrylamide resin (dot-RT). The test was performed at room temperature, with reduced incubation times and with no need for special care in the manipulation of the support. The sensitivity rates obtained were 95.1% for antigen Cc and 97.6% for antigen Cl. Specificity was 90.6% when Cc was used, and 96.9% and 100% when M-Cl and VF-Cl were used, respectively. No significant differences in titer were observed between tests carried out with homologous and heterologous antigens. The low cost and easy execution of the dot-RT test using antigen extracts of *Cysticercus longicollis* indicate the test for use in the immunodiagnosis of human neurocysticercosis.

KEYWORDS: Neurocysticercosis; Dot-ELISA; Cerebrospinal fluid; *Cysticercus cellulosae*; *Cysticercus longicollis*; Resin-treated polyester fabric.

INTRODUCTION

Cysticercosis represents an important public health problem in regions with precarious sanitary conditions. In humans, *Cysticercus cellulosae* has been reported to prefer the central nervous system (CNS), ocular globe and skeletal muscle. Because of the severity of its symptoms, the neurologic form, neurocysticercosis (NC), has been most extensively studied[8,11]. In Brazil, frequencies ranging from 0.2% to 7.5% have been reported, with emphasis on the high cost of patient care and the high mortality and morbidity rates of the disease[8,14].

Under satisfactory immune conditions, the physiopathogenic models of CNS infection are systemic, intermediate and local. NC is the best example of the chronic repetitive local type, with outbreaks of acute recurrence and exacerbation of the immune response detected in cerebrospinal fluid (CSF) in the presence of death and degeneration of the parasite[13]. The symptoms are not characteristic and all neurologic syndromes may be present in NC. These manifestations depend on the number, size, age, vitality, localization, and evolutionary stage of the parasite.

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and also on the inflammatory and immune response of the host. The most frequent clinical manifestations are convulsions, intracranial hypertension, dementia, meningitis, and paraparesis, alone or in combination[21].

The definitive diagnosis of NC is based on clinical, epidemiologic and laboratory criteria (neuroimaging and CSF examination). The methods applicable to the CSF provide data about the inflammatory and immune process[14]. ELISA has been the test most intensely studied in the immunodiagnosis of NC because of its high sensitivity and the advantages for the use of CSF as the sample for investigation[4,7,10,17]. In contrast, the dot-ELISA has been little employed, despite the advantage it offers for use in modestly equipped laboratories[6,18].

Currently available immunologic test for NC diagnosis are highly sensitive, although the difficulty of choosing adequate antigens in sufficient amount continues to exist since naturally infected swine are frequently reared in a clandestine manner and are difficult to handle experimentally. The possibility of choosing an animal model of easy maintenance in the laboratory as an alternative source of parasites for the preparation of adequate antigens was based on the observation that *Taeniae* share common antigens[4,9], as is the case for the larval forms of *Taenia crassiceps*, i.e., *Cysticercus longicollis*. The ORF strain of *Cysticercus longicollis*[5], which reproduces asexually by exogenous budding in the peritoneum of mice, is an important laboratory model because it permits the derivation of antigens for diagnostic use[7,16,19].

The objective of the present study was to develop a dot-ELISA using an N-methylol-acrylamide resin-treated fabric as support[18] and *Cysticercus longicollis* as an alternative antigen source in the immunodiagnosis of NC.

MATERIAL AND METHODS

Parasites

Cysticercus cellulosae, obtained from a naturally infected pig were exhaustively washed in saline solution (0.15 M NaCl) and stored at –20°C in a minimal volume of phosphate buffered saline (PBS, 0.0075 M Na_2HPO_4, 0.0025 M NaH_2PO_4, and 0.14 M NaCl, pH 7.2) containing the following protease inhibitors: PMSF, aprotinin, TLCK, leupeptin and antipain at 2.5 mM concentration (PBS-I). *Cysticercus longicollis*, ORF strain, was maintained by intraperitoneal inoculation of female BALB/c mice. After 90 days the parasites were removed by washing with PBS-I and processed for the preparation of antigen extracts.

Antigen Extracts

Membrane + scolex (M + S) was obtained as described by VAZ & FERREIRA, 1988, with some modifications. *Cysticercus cellulosae* (Cc) were ruptured and centrifuged at 15,000 g for 60 min at 4°C. The sediment was homogenized (Potter, Scientific Glass Apparatus, Inc., USA) on an ice bath and the extract was sonicated (Thorton-Inpec Electronics, Brazil) at 20 kHz, 1 mA, four times for 1 min at 4°C. After slow stirring for 2 h, the extract was centrifuged at 15,000 g for 30 min and the second supernatant represented the M+S-Cc antigen. Vesicular fluid (VF) and soluble membrane (M) proteins were obtained as follows: intact parasites, *Cysticercus longicollis* (Cl), were ruptured and centrifuged at 15,000 g for 60 min at 4°C and the supernatants were sonicated at 20 kHz, 1 mA for four periods of 1 min each on an ice bath. The supernatant obtained after further centrifugation represented the VF-Cl antigen. Membrane (M) antigen was obtained as follows: the sediment from the previous procedure was homogenized and the extract sonicated at 20 kHz, 1 mA for four periods of 1 min at 4°C and submitted to slow stirring for 2 h. After double centrifugation at 15,000 g for 30 min, the second supernatant was obtained, representing the M-Cl antigen. The protease inhibitor pool (0.25 mM) was added to the antigen extracts.

Samples

The CSF samples were divided into two groups: **A**, CSF samples from 41 patients with a diagnosis of NC established according to the clinical, epidemiologic and laboratory criteria used at the Department of Neurology, Faculty of Medicine, University of São Paulo; **B**, control CSF samples from 32 individuals distributed as follows: five patients with neurosyphilis, five patients with bacterial meningitis, seven patients with neurologic symptoms due to AIDS, and 15 apparently normal subjects (patients from a group who had been discharged from the hospital after treatment for bacterial meningitis and with CSF normal).

ELISA

The test was carried out on polystyrene plates using M+S–Cc antigen at the concentration of 10 µg/ml, anti-IgG-peroxidase conjugate (Sigma, USA) and reduced incubation times of 30 min. The cut-off was obtained on the basis of the mean + 2 SD absorbance for the control group.

Dot-ELISA/RT

The assay was carried out as described by VAZ et al., 1990, with modifications in volume and reduction in incubation times. The support used consisted of polyester fabric (Rhodia, Brazil) treated with polymerized NMA resin, 12. 2 g/m^2 [1,3,18]. The antigens were diluted in buffered 0.05 M carbonate-bicarbonate solution, pH 9.6, and fixed to the support (1 µl) by drying at 37°C. After blockade of the reactive sites with Tris-saline blocking

solution (10 mM Tris-hydroxymethylaminoethane and 0.15 M NaCl, pH 7.4, and 0.05% Tween-20) containing 5% skim milk for 30 min, washing with Tris-saline and drying, the CSF samples and their dilutions in blocking solution were added (8 µl) to the dot and the support was again incubated, washed and dried. The anti-human IgG-peroxidase conjugate (Biolab Dagnóstica S/A, Brazil) (20 µl) was added to the dot and the incubation, washing and drying procedures were repeated. The reaction was developed with a chromogen solution of 4-chloro-1-naphthol and 0.5% H_2O_2 in PBS. Positive results were identified on the basis of a purple color in the dots. All incubations were carried out at room temperature in a humid chamber, the washes were performed with slow stirring, and the material was dried with the aid of ventilation.

Statistical Analysis

We calculated the geometric mean of the titers (GMT) for the reactive samples of the group of patients with NC, $n = \log_2$ (titer), and the sensitivity, specificity, agreement and correlation of titers. Data were analyzed by the statistical Q test of Cochran and the McNemar test[12].

RESULTS

Concentrations of 2.5 mg/ml, 2.2 mg/ml and 2.5 mg/ml protein^2 were obtained for the M+S-Cc, M-Cl and VF-Cl extracts, respectively. The ideal concentrations of the M+S-Cc, M-Cl and VF-Cl antigens obtained by bloc titration in the dot-ELISA/RT test were 0.1, 0.2 and 0.1 µg per test, respectively.

Figure 1 shows the dot-ELISA on resin-treated polyester fabric using VF-Cl antigen. Table 1 presents the results obtained, the GMT and the sensitivity and specificity of the assay. The sensitivity and specificity obtained for the dot-ELISA/RT test were 95.1% [95% confidence interval ($Cl_{95\%}$) = 88.3-100] and 90.6% ($Cl_{95\%}$ = 84.9-100) for the M+S-Cc antigens, 97.6% ($Cl_{95\%}$ = 92.8-100) and 96.6% ($Cl_{95\%}$ = 90.8-100) for M-Cl, and 97.6% ($Cl_{95\%}$ = 92.8-100) and 100% ($Cl_{95\%}$ = 96.5-100) for VF-Cl, respectively.

Analysis of agreement and correlation of the titers resulted in 0.96 and 0.92 for M+S-Cc x M-Cl and 0.94 and 0.97 for M+S-Cc x VF-Cl, respectively. Statistical analysis showed no significant difference ($p < 0.05$) between tests or antigens.

DISCUSSION

The investigation of immunodiagnosis in NC, started at the beginning of the century, is of special importance because of the polymorphic aspects of the symptoms and of the chronic repetitive model of the disease, with outbreaks of acute recurrence[14]. Particularly important today is the ELISA[4,7,10,17]. Variations in the results of ELISA are related to the effect of the support on the immobilization of the antigen components, to the conjugate and substrate, to the methods employed for the determination of enzymatic activity, to the samples and dilutions employed, to the procedure used to calculate the cut off, and to the preparation and purification of the antigen extracts. The disadvantages of ELISA are the greater amount of antigen required on the polystyrene support to maintain immunoreactivity and the instability of the final color product, which requires immediate reading. In the dot-ELISA, the reading is visual and the final color product is stable[6,18]. On the other hand, the nitrocellulose membranes of the dot-ELISA have the disadvantage of being more fragile and of difficult manipulation.

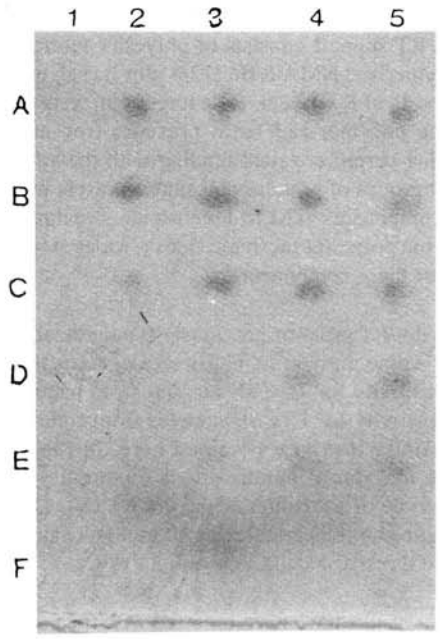

Fig. 1 – Dot-ELISA on N-methylol-acrylamide resin-treated polyester fabric (dot-RT) using antigen of vesicular fluid of *Cysticercus longicollis* (VF-Cl) for the detection of antibodies in the CSF (dilutions 1:2 to 1:32 from A to E) of an apparently normal individual (1) and in the CSF of four patients with neurocysticercosis (2-5). Antigen, conjugate and chromogen controls are in positions 1F, 3F and 5F, respectively.

The dot-ELISA for the immunodiagnosis of NC was developed using a new support of easy preparation and handling and of high stability after binding of the antigen components[3,17], and permitting the use of alternative antigens[7,19].

The RT support consists of polyester fabric treated with polymerized NMA resin (12.2 g/m^2); each m^2 yields 100 supports of 8 x 12 cm, for a total of 96 tests per support. The polymerized resin presents free methylol groups that permit covalent binding with the amine and hydroxyl groups of proteins and sugars present in the antigenic components. The tridimensional structure of the resin on the polyester mesh provides a wider area for the binding of these components[1,18].

The dot/RT test was previously standardized[18] using the total saline antigen of *Cysticercus cellulosae*, with 93.3% positivity for 15 CSF samples from patients, and 100% negativity for 15 CSF samples from controls, i.e., results similar to those obtained here. In the present study we introduced modifications to simplify and reduce the time of execution of the dot/RT test, i.e., incubations at room temperature for 30 min and sample and conjugate directly added to the antigen dot.

Good results have been reported with the use of heterologous antigens in the immunodiagnosis of NC. LARRALDE et al., 1990, using ELISA and *Cysticercus cellulosae* and *Cysticercus longicollis* antigens, obtained sensitivity and specificity of 95% and 96%, 95% and 98%, 97% and 96%, and 92% and 98%, respectively, for the M and VF antigens of the two parasites. In contrast, VAZ et al., 1991, using VF-Cl in the same test, obtained 89% positivity for 28 CSF samples from patients with NC and 100% negativity for CSF samples from 21 control subjects.

In a previous study[16] we characterized M and VF antigen extracts of *Cysticercus longicollis* and concluded that the parasite shares antigenic epitopes with *Cysticercus cellulosae*. Thus, we indicated these parasites as an alternative source for use in the immunodiagnosis of NC. The conditions for antigen preparations are rapid, of simple execution, low cost and feasible for modestly equipped laboratories. M antigens were studied because of the important function of the parasite's tegument with respect to the host. Puncture with a fine gauge needle to obtain VF antigen proved to be unfeasible and of low yield. Thus, we used the method of rupture by pressure and centrifugation, a fact that definitely contaminates the materials with soluble membrane proteins. During the preparation of M extracts, some proteins that are insoluble in aqueous medium may be discarded in the final sediment and chemical extraction studies are needed to clarify this matter.

Almost equivalent concentrations of the M+S-Cc, M-Cl and VF-Cl antigens were used in the dot/RT test. In contrast, ELISA requires ten times higher concentrations, perhaps because of the irreversible binding by adsorption of antigen components to the polystyrene plate and because of the wider area that must be coated with the antigen, different from what occurs with dot-ELISA.

The distribution of titers obtained in the immunologic tests for the CSF samples from patients with NC (Table 1) shows that two samples did not react even though they were reactive in ELISA-Cc, with titers of 2

TABLE 1

Results of the tests applied to 41 CSF samples from patients with NC (A) and to 32 CSF samples from the control group (B), geometric mean of the titers (GMT) and sensitivity (S) and specificity (Sp) indices obtained in the ELISA and dot-ELISA/RT tests according to the antigen used.

Test (antigen)	Group	Non Reactive	Reactive	GMT	Sensitivity (S) Specificity (Sp)
ELISA	A	0	41	143.0	S = 100%
(M+S-Cc)	B	32	0		Sp = 100%
dot/RT	A	2	39	18.0	S = 95.1%
(M+S-Cc)	B	29	3		Sp = 90.6%
dot/RT	A	1	40	12.4	S = 97.6%
(M-Cl)	B	31	1		Sp = 96.9%
dot/RT	A	1	40	17.2	S = 97.6%
(VF-Cl)	B	32	0		Sp = 100%

Cc – *Cysticercus cellulosae*
Cl – *Cysticercus longicollis*
dot/RT – dot-ELISA on resin-treated polyester fabric
M – membrane
M + S – membrane + scolex
VF – vesicular fluid

and 1. The two CSF samples were from patients with the calcified form of NC, and a reduced concentration of antibodies may be observed in these cases[20].

M antigens were responsible for the undesired reactions in the control group, three with the M+S-Cc extract (a CSF sample from a patient with neurosyphilis and two samples from apparently normal individuals), and one with the M-Cl extract (a CSF sample from an apparently normal individual). These cross reactions may be explained by the presence of glycerolphosphorylcholine, reported to be the major membrane component[15], or glycolipid fractions, justifying the better performance of the VF extract, as previously reported[7].

Analysis of GMT (Table 1), of little interest because of the heterogeneous response of patients, shows that ELISA presented the highest results, with most samples showing elevated titers.

Application of the statistical Q test of Cochran and 2 x 2 analysis confirmed the absence of significant differences ($p < 0.05$) between tests or antigens. Similarly, the agreement and correlation indices for the titers demonstrated that the antigens had similar performance, indicating that the dot/RT test using *Cysticercus cellulosae* antigens, VF in particular, can be used as an alternative in the detection of antibodies in the CSF of patients suspected to have NC.

RESUMO

Teste dot-ELISA para detecção de anticorpos anti-*Cysticercus cellulosae* em líquido cefalorraquiano utilizando um novo suporte (tecido de poliéster-resina) e antígenos de *Cysticercus longicollis*

Foi desenvolvido o teste dot-ELISA para detecção de anticorpos em líquido cefalorraquiano (LCR) no diagnóstico imunológico da neurocisticercose humana, utilizando antígenos de membrana e escólex de *Cysticercus cellulosae* (M+E-Cc) e, alternativamente, membrana (M) e líquido vesicular (LV) de *Cysticercus longicollis* (Cl) covalentemente ligados a um novo suporte constituído de tecido de poliéster-resina de N-metilolacrilamida (dot-TR). O teste foi realizado à temperatura ambiente, com tempos de incubação reduzidos e sem necessidade de cuidados na manipulação do suporte. A sensibilidade obtida foi de 95,1% para o antígeno Cc e 97,6% para o Cl. A especificidade foi de 90,6% quando o antígeno Cc foi usado, e 96,9% e 100% para M-Cl e LV-Cl, respectivamente. Não foi observada diferença significativa entre os antígenos homólogo e heterólogo. O baixo custo e a fácil execução do teste dot-TR empregando extratos antigênicos de *Cysticercus longicollis* indicam que pode ser empregado como alternativa no imunodiagnóstico da neurocisticercose humana.

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