

IMMUNOPEROXIDASE FOR THE DETECTION OF ANTIBODIES IN CEREBROSPINAL FLUID IN NEUROCYSTICERCOSIS: USE OF *Cysticercus cellulosae* AND *CYSTICERCUS LONGICOLLIS* PARTICLES FIXED ON MICROSCOPY SLIDES

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SUMMARY

The ORF strain of *Cysticercus longicollis* represents an important model for the study of heterologous antigens in the immunodiagnosis of neurocysticercosis (NC). The immunoperoxidase (IP) technique was standardized using a particulate antigen suspension of *Cysticercus longicollis* (Cl) and *Cysticercus cellulosae* (Cc). Cerebrospinal fluid (CSF) samples were incubated on the antigen fixed to microscopy slides; the conjugate employed was anti-IgG-peroxidase and the enzymatic reaction was started by covering the slides with chromogen solution (diaminobenzidine/H₂O₂). After washing with distilled water, the slide was stained with 2% malachite green in water. Of the CSF samples from 21 patients with NC, 19 (90.5%) were positive, whereas the 8 CSF samples from the control group (100%) were negative. The results of the IP-Cl test applied to 127 CSF samples from patients with suspected NC showed 28.3% reactivity as opposed to 29.1% for the IP-Cc test. The agreement index for the IP test (Cl x Cc) was 94.2%, with no significant difference between the two antigens.

KEYWORDS: Immunoperoxidase; Neurocysticercosis; *Cysticercus cellulosae*; *Cysticercus longicollis*; Cerebrospinal fluid.

INTRODUCTION

Human NC, caused by the larval form of *Taenia solium*, *Cysticercus cellulosae*, is the most extensively studied parasitic infection of the central nervous system, especially in regions where poor sanitation conditions prevail. The detection of specific antibodies in CSF is considered one of the auxiliary criteria for diagnosis¹¹ and several immunological methods have been standardized. The introduction of immunofluorescence^{7,9} and

immunoenzymatic^{2,12,13} methods has increased the efficiency of antibody detection in CSF from patients with NC. A limitation of these methods is the obtention of antigens from naturally infected swine in appropriate amounts to guarantee the homogeneity of antigen lots. As an alternative antigen source, the mutant strain ORF of the larval form of *Taenia crassiceps*, *Cysticercus longicollis*⁵, which reproduces asexually by exogenous

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budding in the peritoneum of mice, has been studied^{6,14,15}.

In the present study we standardized the IP test for the detection of antibodies in the CSF using a particulate *Cysticercus longicollis* antigen and compared its results with those obtained with *Cysticercus cellulosae* antigens.

MATERIAL AND METHODS

Parasites

Cysticercus longicollis, ORF strain was maintained by intraperitoneal passage in female BALB/c mice aged 8 to 12 weeks. Five parasites, small forms with no visible buds, were inoculated with the aid of a 25 x 7 gauge needle and a minimal volume of phosphate buffered saline, PBS (0.0075 M Na₂HPO₄, 0.0025 M NaH₂PO₄, and 0.14 M NaCl, pH 7.2). The animals were sacrificed 90 days later, the parasites were removed from the peritoneal cavity with PBS containing a pool of 2.5 x 10⁻³ mM protease inhibitors (PBS-I) and stored at -20°C after exhaustive washings in saline solution.

Intact *Cysticercus cellulosae* parasites obtained from muscles of naturally infected swine were exhaustively washed in saline solution (0.15 M NaCl) and those in the process of degeneration or calcified were discarded. The parasites were stored at -20°C in a minimum volume of PBS-I. The inhibitors (Sigma Chem. Co., St. Louis, MO, USA) used were PMSF (phenylmethylsulfonyl fluoride), aprotinin, leupeptine, antipain and TLCK (N-p-tosyl-lysine-chlormethylketone).

Particulate antigen

The parasites were thawed, homogenized in a Potter homogenizer and lyophilized. The product obtained was slightly ground in a mortar and stored at 4°C in a desiccator. The antigen suspension, 1.5 mg/ml in PBS, was fixed (10 µl per area) by drying at 37°C for 2 hours on microscope slides that were stored and protected from humidity at -20°C for up to 3 months.

Immunoperoxidase test

The CSF samples (15 µl) were incubated on the antigens in a humid chamber at 37°C for two hours and at 4°C for an additional 18 hours. After three washes in PBS (5 minutes each) and drying, the slides were incubated with anti-human IgG-peroxidase conjugate (Biolab Diagnóstica SA, Brazil) diluted in PBS according to titer for two hours at 37°C, and the washing procedure was repeated. The enzymatic reaction was started by covering the slide with chromogen solution (3.5 mg/ml diaminobenzidine and 1 µl/ml H₂O₂ in PBS) with shaking for 5 minutes and stopped by washing the slide with distilled water. For better contrast, the slide was counterstained with a 2% aqueous solution of malachite green.

After drying, the slides were mounted in glycerin, pH 7.2. The slides were examined with a common light microscope (100x and 400x) and antigen particle staining was observed as an intense brown precipitate in positive samples and as a greenish color in negative test.

CSF samples

A total of 156 CSF samples divided into three groups were examined: Group A – 21 samples from patients with confirmed NC according to the criteria of the Department of Neurology, Faculty of Medicine, University of São Paulo; Group B – 8 samples from patients with neurologic disorders but with no NC; Group C – 127 samples from patients with clinically-epidemiologically suspected NC. All samples were tested by ELISA¹⁰ using whole saline extract of *Cysticercus cellulosae*¹³.

RESULTS

Fig. 1 shows the appearance of positive and negative CSF in the IP test using particulate antigens of Cc and Cl. Table 1 shows the results obtained in the IP-Cc and IP-Cl tests for the 156 CSF samples studied, compared to ELISA. Of the 21 CSF samples from patients with NC, 19 (90.5%) were positive and the 8 samples from the control group were negative. Of the 127 CSF samples from group C, 40 (31.5%) were positive in the ELISA, 37 (29.1%) were positive in the IP-Cc test and 36 (28.3%) were positive in the IP-Cl test. The agreement indices obtained were 89.7% for ELISA x IP-Cl and 94.2% for ELISA x IP-Cc and IP-Cc x IP-Cl.

DISCUSSION

The detection of anti-*Cysticercus cellulosae* antibodies in the CSF is one of the diagnostic tools used in NC. The present study was motivated by the difficulty in obtaining homologous antigens and by the need to increase the efficiency of immunodiagnosis in NC.

In view of the complexity of the parasite and the chronic nature of the disease, when elaborating a diagnostic test for the detection of specific antibodies one should consider the genetic diversity of the host and the temporal variations in the evolution of the parasite and of the infection itself. The probability of obtaining an antigen fraction universally recognized by all hosts throughout the course of infection is reduced¹⁵.

The use of *Cysticercus longicollis* antigens, ORF strain, in the form of lyophilized particles was standardized to provide an alternative source for the IP test compared to the use of *Cysticercus cellulosae*. The execution of the test under standardized conditions resulted in a clear discrimination between positive and negative samples (Fig. 1). In contrast to what we had

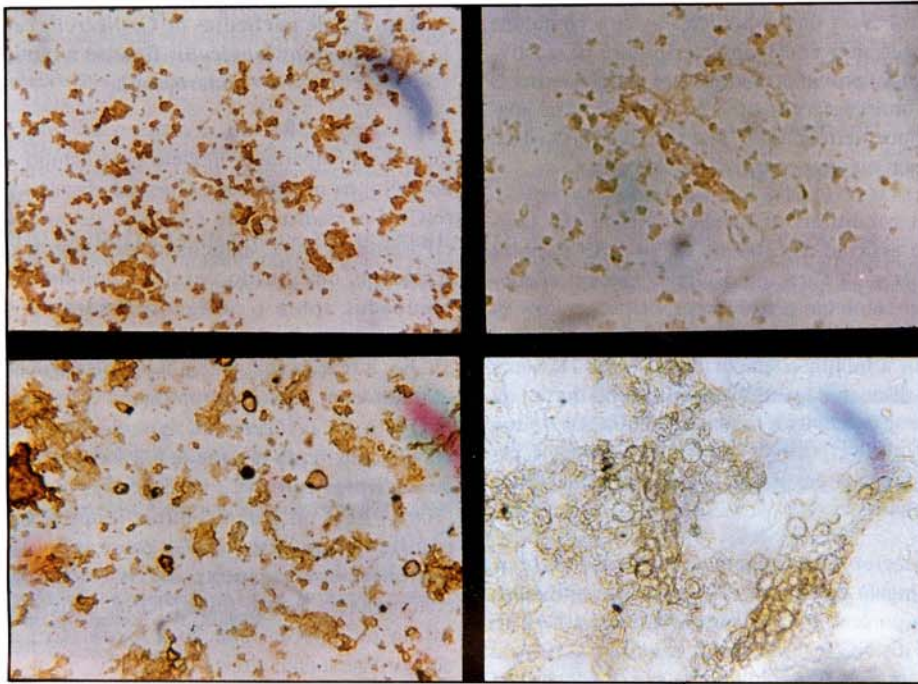


Fig. 1: Immunoperoxidase test for the detection of antibodies in CSF from neurocysticercosis patient (left) and from control group individual (right), using *Cysticercus cellulosae* (top) and *Cysticercus longicollis* (bottom) particles fixed on microscopy slides.

observed earlier¹⁰, it was not necessary to pretreat the antigen extracts with acetone to remove lipid components. Indeed, this treatment resulted in lower reactive intensity which led us to suppress it (not shown data). On the other hand, the presence of phosphorylcholine

has been detected in the membrane of these parasites, often nonspecifically recognized by serum immunoglobulins¹. This nonspecificity was not observed, possibly because we used CSF and because of the lower sensitivity of the IP test.

TABLE 1

Results obtained for the CSF samples from patients with neurocysticercosis (A), from the control group (B) and from the screening group (C) by ELISA and immunoperoxidase test (IP) according to the antigen used.

Test	Group	n	R	NR	Positivity index	Negativity index	Agreement index
ELISA*	A	21	21	0	100%	100%	ELISA x IP-Cc 94.2%
	B	8	0	8			
	C	127	40	87	31.5%		
IP-Cl	A	21	19	2	90.5%	100%	ELISA x IP-Cl 89.7%
	B	8	0	8			
	C	127	36	91	28.3%		
IP-Cc	A	21	19	2	90.5%	100%	IP-Cc x IP-Cl 94.2%
	B	8	0	8			
	C	127	37	90	29.1%		

*Total saline extract of *Cysticercus cellulosae*
 Cc, antigenic particles of *Cysticercus cellulosae*
 Cl, antigenic particles of *Cysticercus longicollis*
 R, reactive
 NR, nonreactive

The control assay on the particles using a conjugate and a chromogen showed a complete absence of reactivity, indicating that previous treatment of antigen extracts with H_2O_2 is unnecessary for the neutralization of possible endogenous peroxidase¹⁴. The dehydration caused by lyophilization may have neutralized this effect.

Different conditions of incubation time and temperature were assayed. Incubation of the CSF samples for only 2 hours at 37°C required a larger amount of conjugate in order to obtain proper reactivity, especially in the case of *Cysticercus longicollis* particles. Incubating the samples in a humid chamber at 37°C for 18 hours presented the disadvantage of evaporation and drying on the slide in some cases, with immunoglobulin adsorption and consequent false-positive results. So, it was necessary to incubate the slides at 4°C in a humid chamber for additional 18 hours.

The satisfactory results obtained with the anti-human IgG conjugate confirmed that IgG class antibodies are frequently present in high concentrations in the CSF of patients with NC^{3,8}. The IP test required a 10-fold higher concentration of conjugate, possibly because of the lower accessibility to the particulate antigen compared to the solubilized antigens used in the ELISA. We are currently investigating the use of biotinylated conjugates that, by expanding peroxidase binding, may obviate this drawback.

We also assayed the use of the chromogen 4-chloro-1-naphthol¹³, producing a purple precipitate of less contrast upon microscopic examination. When used as a background stain for 30 seconds, 2% hematoxylin presented satisfactory results, although the use of malachite green apparently produced a better contrast in positive tests.

The results obtained (Table 1), 100% negativity for the control group, 90.5% positive for the group of NC patients and 89.7% agreement with ELISA using a homologous antigen, are similar to those obtained by the indirect immunofluorescence test¹⁰, suggesting that the determining factor in both methods may be antigen accessibility⁴. The advantage of the IP test is that it can be read using a common light microscope⁴.

The IP test using a particulate antigen of *Cysticercus longicollis* or *Cysticercus cellulosae* could be used as an alternative for the detection of antibodies in the CSF in the immunodiagnosis of NC.

RESUMO

Imunoperoxidase para detecção de anticorpos em líquido cefalorraquiano na neurocisticercose:

emprego de partículas de *Cysticercus cellulosae* e *Cysticercus longicollis* fixadas a lâminas de microscopia

A cepa ORF de *Cysticercus longicollis* (Cl) representa importante modelo para estudo de antígenos heterólogos no imunodiagnóstico da neurocisticercose (NC). Foi padronizada a técnica de imunoperoxidase (IP) empregando suspensão antigênica particulada. Amostras de líquido cefalorraquiano (LCR) foram incubadas sobre o antígeno fixado em lâminas de microscopia, o conjugado empregado foi anti-IgG-Peroxidase, a reação enzimática iniciou-se ao cobrirem-se as lâminas com solução cromógena (Diaminobenzidina/ H_2O_2). Após lavagens em água destilada, a lâmina foi corada com verde malaquita a 2% em água. De 21 LCR de pacientes com NC, 19 (90,5%) foram reativos e 8 (100%) LCR do grupo controle foram não reativos. Os resultados do teste IP-Cl ensaiando 127 LCR de pacientes com suspeita de NC mostrou 89,7% de concordância com o teste ELISA empregando extrato salino de *Cysticercus cellulosae* (Cc) e 94,2% de concordância com o teste IP-Cc.

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