

HEMAGGLUTINATION TEST FOR THE DIAGNOSIS OF HUMAN NEUROCYSTICERCOSIS: DEVELOPMENT OF A STABLE REAGENT USING HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

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SUMMARY

A hemagglutination (HA) test was standardized using formalin- and tannin-treated gander red blood cells sensitized with a total salt extract of *C. cellulosae* (HA-Cc) and an antigenic extract of *Cysticercus longicollis* (HA-CI) vesicular fluid. A total of 61 cerebrospinal fluid (CSF) samples were assayed, 41 from patients with neurocysticercosis and 20 from a control group which were, respectively, reactive and non-reactive to ELISA using *C. cellulosae*. The CSF samples from the control group did not react and 35 (85.4%) and 34 (82.9%) CSF samples from patients were reactive to the HA-Cc and HA-CI tests, respectively. The reagents ready for use were stable up to 6 months when stored at 4°C in 50% glycerol. The present results confirm that the reagent using *Cysticercus longicollis* stabilized with glycerol can be used as an alternative in the immunological diagnosis of neurocysticercosis.

KEYWORDS: Hemagglutination test; Neurocysticercosis; Cerebrospinal fluid.

INTRODUCTION

Neurocysticercosis (NC), an infection with the larval form of *Taenia solium*, *Cysticercus cellulosae*, is the most frequent parasitic infection of the central nervous system occurring in countries with inadequate sanitation or receiving immigrants from endemic regions¹⁰.

The clinical symptoms are not specific and the diagnosis is made with the aid of neuroimaging and cerebrospinal fluid (CSF) examination. Immunological methods for the detection of antibodies in the CSF have been extensively employed since the location of the parasite limits a direct diagnosis¹⁵. Despite the difficulty in obtaining homogeneous and stable lots of sensitized red blood cells, indirect hemagglutination (HA) continues to be an important method because of its easy execution, low cost and relative efficacy.

A limiting factor for the standardization of reagents for the diagnosis of NC has been the derivation of parasites from infected swine. The cross-reactions observed between the antigens

of *Taenia solium* and *Taenia crassiceps*, ORF strain, cysticerci⁴ indicate that the parasites share important epitopes for use as sources of antigen in the immunological diagnosis of NC^{1,6,8,18,19}.

The objective of the present study was to develop an HA test for the detection of antibodies in the CSF of patients with NC using *Taenia crassiceps* cysticerci, avian red blood cells and stabilizers for the final reagent.

MATERIAL AND METHODS

Cysticerci

Taenia crassiceps cysticerci, *C. longicollis* ORF strain, was kindly supplied by Dr. C. Larralde (Instituto de Investigaciones Biomedicas, Universidad Nacional Autónoma de México). Parasites were 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 × 7 mm gauge needle and 0.1 ml 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

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removed from the peritoneal cavity with PBS containing a pool of 2.5×10^{-3} mM protease inhibitors (PBS-I) and stored at -20°C after exhaustive washings in saline solution (0.15 M NaCl). The inhibitors (Sigma Chem. Co.; St. Louis, USA) used were PMSF (phenylmethylsulfonyl fluoride), aprotinin, leupeptine, antipain and TLCK (N-p-tosyl-L-lysine-chloromethylketone).

Taenia solium intact metacestodes obtained from muscles of naturally infected swine were exhaustively washed in saline solution 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.

Antigens

Whole salt extract of *Taenia solium* metacestodes (Cc): 200 parasites were homogenized in 2 ml of distilled water and the mixture was sonicated at 3 cycles of 2 minutes each, at 1 mA and 20 kHz. After the addition of 2 ml 0.3 M NaCl and further sonication, the extract was centrifuged at 15,000 g for 60 minutes at 4°C and the supernatant obtained was again centrifuged. The pool of protease inhibitors was added to the second supernatant at 0.25 mM concentration.

Vesicular fluid from *Taenia crassiceps* cysticerci (CI): 800 whole parasites were ruptured with the aid of a glass rod and centrifuged at 15,000 g for 60 minutes at 4°C . The supernatant was sonicated, centrifugation was repeated and the inhibitor pool was added to the supernatant obtained. The antigens were divided into aliquots and stored at -20°C .

The antigens were characterized in terms of protein concentration⁹ and peptide composition by SDS-PAGE under reducing conditions using a discontinuous system of buffers on 12% gel^{7, 16}.

Hemagglutination (HA) test

An equal volume of 10% p-formaldehyde in PBS was added to a 10% (v/v) adult gander red blood cell saline solution and the mixture was kept at 37°C for 18 hours with occasional shaking. The cells were centrifuged, washed four times in saline (2000 rpm for 3 minutes), resuspended at 20% concentration in PBS containing 4% p-formaldehyde (w/v) and stored at 4°C for up to 2 months.

Red blood cells were sensitized by the method of HOSHINO-SHIMIZU et al.⁵ with some modifications. Two ml aliquots of 1.5% red blood cells in saline were washed three times and resuspended in the original volume. An equal volume of 1:10,000 tannic acid in saline (w/v) was added, the mixture was incubated for 15 minutes at 56°C and resubmitted to the washing procedures. Two ml of the antigen diluted in PBS 6.4 (0.05 M KH_2PO_4 , 0.025 M NaH_2PO_4 and 0.075 M NaCl, pH 6.4) were added to the red cell concentrate and the mixture was incubated for 50 minutes at 37°C . Two ml 0.1% glutaraldehyde in saline was then added and the mixture was further incubated for 30 minutes. The red cells were washed and resuspended in stabilizing solution of the following composition: 1.3% glycine, 0.03%

sodium thioglycolate, 0.03% merthiolate, 0.9% skim milk, and 0.7% sodium glutamate in PBS 6.4 (Eo). Control red cells with no antigen added during the sensitization process were similarly prepared. We assayed 1% to 3% red cell suspension and antigen concentrations of 15 to 75 $\mu\text{g}/\text{ml}$ by bloc titration. The test was carried out on V-bottom polystyrene microtiter plates (Inlab, Brazil) by homogenizing 50 μl of the CSF samples and their serial dilutions in PBS 6.4 starting from 1:2, and 50 μl of sensitized red cells. Readings were taken after 45 minutes at room temperature.

Three conditions for the storage of ready to use reagents were assayed: Eo, E1 (50% glycerol in Eo) and E2 (40% ethyleneglycol in Eo). Silicone (0.08%) was added to the solutions to minimize the foaming effect. Glycerol and ethyleneglycol were selected on the basis of reports of their use for the preservation of human sera^{2,3} and of hemagglutination reagents¹³. Aliquots of the stabilized reagent were stored at 4°C and 37°C and assayed at 15 day intervals using positive and negative standard CSF.

CSF samples

A total of 61 CSF samples divided into two groups were assayed: Group A – 41 samples from patients with confirmed NC according to the criteria of the Department of Neurology, Faculty of Medicine, University of São Paulo. The criteria were clinical syndromes, positive computed tomography and the CSF samples were reactive to the ELISA using whole saline extract of *Cysticercus cellulosae*¹². Group B – 20 samples from patients with neurologic disorders but with no NC (9 apparently normal subjects, 4 with epilepsy, 2 with neurosyphilis and 5 with bacterial meningitis), all of them negative to ELISA.

RESULTS

The protein concentrations obtained for the Cc and CI antigens were 3.45 mg/ml and 3.20 mg/ml, respectively. Analysis of the SDS-PAGE data showed the presence of bands of 98-94 kDa, 72-68 kDa, 46-44 kDa, 40-38.5 kDa, 37-34 kDa, 32-30 kDa and 27-26 kDa in the Cc antigen, and of bands of 115-108 kDa, 95-92 kDa, 70-68 kDa, 41 kDa, 38.5-36 kDa, 26 kDa, and 10-15 kDa (diffuse) in the CI antigen (Figure 1).

The best appearance of the hemagglutination test was observed with 1.5% red cells and a final reading after 45 minutes for the Eo stabilizer and after 60 minutes for E1 and E2. The ideal concentrations of the Cc and CI antigens were 35 and 30 $\mu\text{g}/\text{ml}$ reagent, respectively.

Nonspecific agglutinins were not observed in the test with control red cells. Of the 41 CSF samples from the patient group, 35 (85.4%) and 34 (82.9%) reacted with antigens Cc and CI, respectively, with geometric mean titers of 18.0 ± 6.8 in the HA-Cc test and of 11.4 ± 6.1 in the HA-CI test. No reactivity was observed in the CSF samples from the control group. The agreement indices obtained were HA-Cc \times HA-CI = 98.3%, ELISA \times HA-CI = 88.5%, and ELISA \times HA-Cc = 90.2%. Analysis of the results by the Cochran Q test and by the McNemar test¹⁴

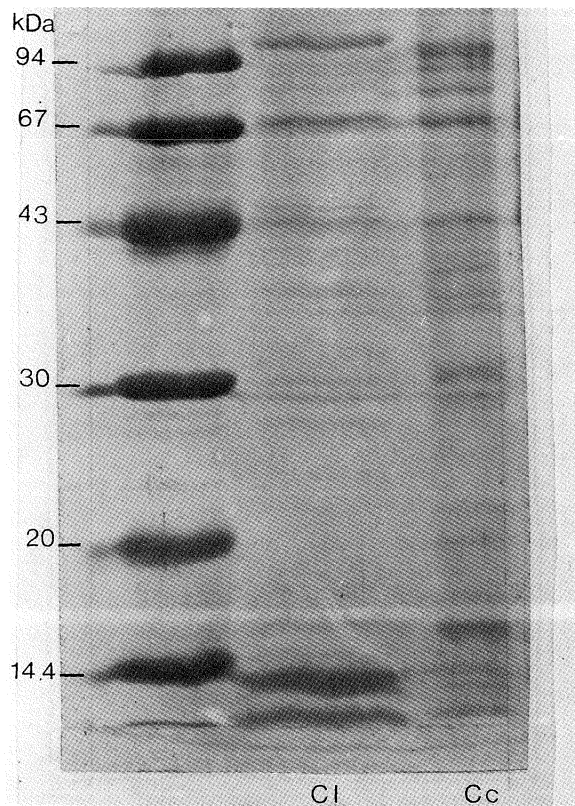


Fig. 1 – Polyacrylamide 12% gel electrophoresis applied to antigens from *Taenia solium* (Cc) and *Taenia crassiceps* (CI) cysticerci. Molecular weight markers are given on the left (kDa).

confirmed the advantage of ELISA and showed no significant difference between the Cc and CI antigens in the HA test.

The stability assay for the reagent showed the advantage of the use of 50% glycerol and of storage at 4°C for up to 6 months (Figure 2).

DISCUSSION

The objective of the present study was to standardize the HA test using formalin- and tannin-treated gander red blood cells, obeying the recommendations of the Health Ministry not to use human blood derivatives for the production of reagents¹¹. SASAKI et al., 1996, described the hemagglutination test for the diagnosis of American trypanosomiasis using gander red blood cells.

We used a vesicular fluid antigen from *Taenia crassiceps* cysticerci, ORF strain, maintained in laboratory animals and compared it to the use of homologous antigen.

The concentration used of 60 µg CI per 2 ml red cells (1.5 µg per test) was similar to that of the Cc antigen. This concentration is variable and depends on the choice of red cells, antigen and sensitization conditions^{12,17}. In the process of antigen characterization (Figure 1) we observed the peptides reported to be immunodominant in Cc and CI¹⁹.

The CSF samples from the control group were 100% negative. In the group of NC patients, seven CSF samples (17.1%) did not react to the HA-CI test and six (14.6%) did not react to the Cc antigen, values that correspond to satisfactory sensitivity rates. False-negative results have been observed in all immunological tests for NC^{1,12,19}. The standardized HA test is of simple execution and low cost, especially for less complex laboratories.

The present results were similar to those reported in the literature. UEDA et al.¹⁷ using the Cc antigen, obtained sensitivity and specificity of 88.7% and 96.6% with sheep red cells and of 81.7% and 94.4% with human red cells, respectively. PIALARISSI et al.¹² applied the test to 125 CSF samples from patients with NC and obtained 87.2% positivity, a rate comparable to that of indirect immunofluorescence, with the advantage of lower cost and easier execution.

The addition of glycerol to the stabilizing solution increased the stability of the reagent up to 6 months when stored at 4°C (Figure 2). Either SASAKI et al.¹³ obtained best results using glycerol, but just for 90 days at 4°C. Although glycerol increases the viscosity of the medium, the use of nucleated gander red cells maintained an appropriate time for the final reading. Other stabilizing solutions will be evaluated later.

No significant difference was observed between antigens, indicating that antigens of *Taenia crassiceps* metacestodes can be used as an alternative in the HA test for the detection of antibodies in the CSF of patients with NC.

RESUMO

Teste de hemaglutinação no imunodiagnóstico da neurocisticercose humana: desenvolvimento de reagente estável utilizando antígenos homólogo e heterólogo

Foi padronizado o teste de hemaglutinação (HA) utilizando hemácias formolizadas e taninizadas de ganso sensibilizadas com extrato salino total de *C. cellulosae* (HA-Cc) e líquido vesicular de *Cysticercus longicollis* (HA-CI). Foram ensaiadas 61 amostras de líquido cefalorraquiano (LCR), 41 de pacientes com neurocisticercose e 20 de um grupo controle, respectivamente, reagentes e não-reagentes no teste ELISA utilizando antígenos de *C. cellulosae*. As amostras do grupo controle foram não-reagentes e 35 (85,4%) e 34 (82,9%) LCR dos pacientes foram reativos nos testes HA-Cc e HA-CI, respectivamente. O reagente de hemácias sensibilizadas prontas para uso, foi estável por até 6 meses quando armazenado a 4°C em solução estabilizante com 50% de glicerol. Os resultados confirmam que o reagente de HA-CI pode ser empregado como alternativa no diagnóstico imunológico da neurocisticercose.

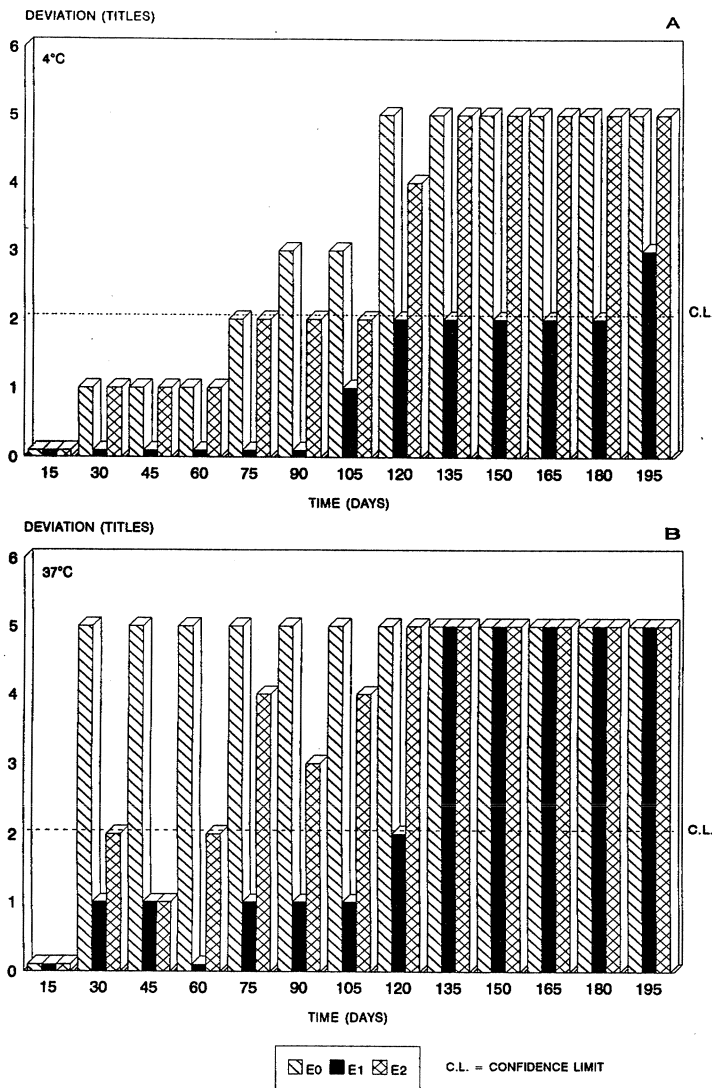


Fig. 2 – Assay of the stability of the sensitized red cell reagent in liquid form ready for use. The three stabilizer conditions [E0, E1 (E0 + glycerol) and E2 (E0 + ethyleneglycol)] were assayed at 4°C (A) and 37°C (B). The confidence limit was considered as a maximum deviation of two titles from the expected result.

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