**Taenia saginata** metacestode antigenic fractions obtained by ion-exchange chromatography: Potential source of immunodominant markers applicable in the immunodiagnosis of human neurocysticercosis

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**A R T I C L E   I N F O**

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**A B S T R A C T**

The aim of this study was to fractionate and partially characterize fractions obtained from the total saline extract (SE) of *Taenia saginata* metacestodes after ion-exchange procedure in carboxymethyl sepharose (CM) and diethylaminoethyl sepharose (DEAE) resins, as a source of antigenic markers applicable in the immunodiagnosis of neurocysticercosis (NCC). For IgG detection by enzyme-linked immunosorbent assay (ELISA) and immunoblotting, 140 serum samples were analyzed: 45 from patients with NCC (G1), 50 from patients with other parasitic infections (G2), and 45 from healthy individuals. Sensitivity (Se), specificity (Sp), area under curve (AUC), and likelihood ratios (LR) were calculated. CM S2 and DEAE S2 fractions provided high diagnostic values (Se 88.8% and 93.4%; Sp 93.7% and 92.6%; AUC 0.965 and 0.987; LR+ 14.07 and 12.67; LR−0.11 and 0.07, respectively). In conclusion, CM S2 and DEAE S2 fractions are important sources of specific peptides, with high efficiency to diagnose NCC.

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1. Introduction

The diagnosis of neurocysticercosis (NCC), the most common parasitic infection of the human central nervous system, is established by the combined analysis of clinical data, neuroimaging (computed tomography and magnetic resonance), immunologic, and epidemiologic data. Neuroimaging methods can be inaccessible and/or too expensive in many countries where *Taenia solium* is endemic. Serologic tests as enzyme-linked immunosorbent assay (ELISA) and immunoblotting to detect specific antibodies against metacestodes of *T. solium* in serum samples or cerebrospinal fluid (CSF) may provide a viable tool for the diagnosis of NCC (Barcelos et al., 2007; Del Brutto, 2012a; Esquivel-Velázquez et al., 2011; Foyaca-Sibat et al., 2009; Handali et al., 2010; Sahu et al., 2009).

The use of heterologous antigen from *Taenia saginata* metacestodes for NCC immunodiagnosis is important where the collection of *T. solium* metacestodes is difficult. It is complex to obtain parasites from naturally infected pigs for the preparation of *T. solium* homologous antigen in nonendemic countries; thus, alternative antigens, including heterologous antigens from *T. saginata*, have been used with satisfactory results to diagnose human NCC. *T. saginata* metacestodes can be obtained in considerable quantities, due to the habitual diet in many countries, such as Brazil, mainly based on beef consumption (Oliveira et al., 2007, 2009; Sako et al., 2006). Fractionation of heterologous antigen from *T. saginata* metacestodes allows selection of reactive antigenic fractions potentially applicable in NCC immunodiagnosis, avoiding problems of cross-reactivity with sera from patients infected with other helminthic diseases (Gonçalves et al., 2010; Nunes et al., 2010; Oliveira et al., 2010; Ribeiro et al., 2010).

The aim of this study was to fractionate total saline extract (SE) of *T. saginata* metacestodes in ion-exchange resins, carboxymethyl sepharose (CM) and diethylaminoethyl sepharose (DEAE), to obtain antigenic fractions with potential application in the immunodiagnosis of NCC.

2. Material and methods

2.1. Serum samples

This study was conducted according to the ethical guidelines of the Brazilian Health Ministry after being approved by the Research Ethics Committee of the Universidade Federal de Uberlândia, state of Minas Gerais, Brazil. Serum samples were collected from 140 subjects who...
attended the Laboratory of Clinical Analysis of the Clinical Hospital from the Universidade Federal de Uberlândia (Groups 1 and 2) and from the Laboratory of Parasitology (Group 3) of the Universidade Federal de Uberlândia, and maintained in the Biological Samples Bank of Laboratório de Diagnóstico de Parasitoses.

Group 1 (G1) consisted of 45 patients with definitive diagnosis of NCC: a) all patients presented at least 1 type of clinical manifestation suggestive of NCC such as epilepsy, cephalaea, dizziness, dementia, faintness, hydrocephalus, and no signs or symptoms of cysticercosis in other organs; b) all patients came from or lived in an area where cysticercosis is endemic; c) for immunodiagnosis, cerebrospinal fluid and/or serum samples were positive in an ELISA test for the detection of IgG anti-metacestodes of T. solium; d) they presented evidence of the parasite in neuroimaging, with the following characteristics: active NCC (n = 25), where metacestodes are viable or in early degeneration, and inactive NCC (n = 20), where metacestodes are completely degenerated, classified according to Sotelo et al. (1985).

All patients from Group 1 have a definitive diagnosis, with 25 (55.5%) of them having absolute criteria and 20 (44.5%) with 2 major criteria plus 1 minor or 1 epidemiologic criterion, according to Del Brutto (2012b).

Group 2 (G2) consisted of 50 patients with other parasitic infections: Ascaris lumbricoides (n = 6), Enterobius vermicularis (n = 5), hookworm (n = 5), Giardia lamblia (n = 4), Hymenolepis nana (n = 4), Schistosoma mansoni (n = 4), Taenia sp. (n = 6), Strongyloides stercoralis (n = 7), and Trichuris trichiura (n = 4). Co-infected patients were also tested: hookworm + A. lumbricoides + T. trichiura (n = 1), hookworm + A. lumbricoides (n = 1), Entamoeba histolytica + G. lamblia (n = 1), and H. nana + E. vermicularis (n = 1). Three fecal samples were tested by the parasitologic method of Ritchie (1948), established by demonstrating the parasitic forms in stool examinations.

Group 3 (G3) was composed of 45 healthy individuals, based on their clinical presentation. Although they came from areas where cysticercosis is endemic, no volunteers from this group presented evidence of household contact with T. solium infection or a history of taeniasis or cysticercosis. In addition, 3 fecal samples from these individuals tested negative by the parasitologic methods of Baermann (1917) and Lutz (1919). It means that they were healthy and showed no clinical manifestations suggestive of other infections.

2.2. Animals and parasites

T. saginata metacestodes were obtained from naturally infected bovines in the Triângulo Mineiro region, State of Minas Gerais, Brazil. Infected cattle came from abattoirs and were slaughtered in accordance with the inspection technique recommended by the Federal Inspection Service. T. saginata metacestodes were obtained by dissecting muscles; only those in the vesicular stage were collected, washed in saline solution (0.15 mol/L NaCl) 4 times, and stored at −20 °C.

2.3. Preparation of saline extract (SE) from T. saginata metacestodes

The total SE from 50 metacestodes of T. saginata was resuspended in 5 mL of distilled water containing protease inhibitors (ethylene-diaminetetraacetic acid 1 mmol/L, benzamidine 1 mmol/L, phenyl methyl sulfonyl fluoride 1 mmol/L, aprotonin 1 μg/mL, and leupeptin 2 μg/mL). The metacestodes were homogenized using a glass tissue homogenizer at 4 °C for 5 min and then submitted to 4 sonicating cycles in an ice bath at 40 kHz for 30 s each; after that, 5 mL of 0.3 mol/L NaCl was added, and the mixture was again submitted for sonic treatment, kept at 4 °C for 2 h, and submitted for centrifugation at 12,400 × g for 30 min at 4 °C. The supernatant was assayed according to Lowry et al. (1951) for protein quantification and stored at −20 °C.

2.4. Ion-exchange chromatography

Fractions of total SE were obtained by ion-exchange chromatography developed in microtubes according to Gonzaga et al. (2013) Briefly, 1200 μg of SE was loaded onto a 200-μL carboxymethyl sepharose (CM) resin and diethylaminoethyl sepharose (DEAE) resin (GE Healthcare Life Sciences, Buckinghamshire, UK), previously equilibrated with 10 volumes of phosphate-buffered saline (PBS) in 3 cycles of centrifugation (2000 × g, 2 min). The suspension SE/resins were maintained by gently mixing for 20 min at 4 °C. Afterwards, the suspensions were centrifuged and the supernatants were recovered and considered as nonbinding resin fractions (CM S1 and DEAE S1). Resins were washed 3 times, followed by cycles of centrifugation with 10 volumes of PBS, and the retained proteins (CM S2 and DEAE S2) were eluted using PBS supplemented with 0.5 mol/L of NaCl. The fractions obtained were assayed according to Lowry et al. (1951) for protein quantification and stored at −20 °C. SE and its fractions (CM S1 and CM S2, DEAE S1 and DEAE S2) were analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions, as described by Laemmli (1970). Proteins were visualized in SDS-PAGE after silver staining (Friedman, 1982).

2.5. ELISA to detect specific IgG anti–T. solium metacestodes

Preliminary experiments were carried out to determine the optimal conditions for ELISA, through block titration of reagents (antigens, sera, and conjugate). Polystyrene microplates of low affinity (Interlab, Brazil) were coated with each antigen (SE, CM S1, CM S2, DEAE S1, or DEAE S2) (5 μg/mL) in carbonate bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Microplates were washed 3 times, for 5 min each time, with PBS containing 0.05% Tween 20 (PBS-T). Then serum samples were diluted 1:200 in PBS-T containing 3% of nonfat milk (PBS-T-M) and incubated for 45 min at 37 °C. After another step of washing, the immunoenzymatic conjugate (peroxidase-goat anti-human IgG, Fc specific; Sigma, St. Louis, MO, USA) was added, diluted 1:2000 in PBS-T, and incubated for 45 min at 37 °C. The assay was developed by adding the enzyme substrate consisting of hydrogen peroxide and o-phenylenediamine (OPD) in 0.1 mol/L citrate phosphate (Na2HPO4) buffer (pH 5.5) for 15 min. Reaction was interrupted with the addition of 25 μL per well of 2N H2SO4. Absorbance was determined at 492 nm in an ELISA plate reader (TiterTek Plus, Flow Laboratories, USA).

2.6. Electrophoretic transfer and immunoblotting

The SE and its fractions (CM S1, CM S2, DEAE S1, and DEAE S2) were submitted to SDS-PAGE. Then gels were transferred to nitrocellulose membranes (0.20 μm; Bio-Rad, Hercules, CA, USA), as described by Towbin et al. (1979), using a Trans-Blot SD Semy Dry Electrophoretic Transfer Cell (Bio-Rad). The immunoblotting was performed as described by Nunes et al. (2010), with modifications. The nitrocellulose strips containing SE and its fractions (CM S1, CM S2, DEAE S1, or DEAE S2) were blocked with 5% of nonfat milk in PBS-T for 2 h at room temperature and incubated overnight at 4 °C with serum samples diluted 1:50 in 1% of nonfat milk in PBS-T (PBS-TM). After washing with PBS-TM, the strips were incubated for 2 h at room temperature with the conjugate peroxidase–labeled goat anti-human IgG (whole molecule, Sigma) diluted at 1:1500 in PBS-TM. The strips were washed in PBS, and the assay was developed by adding hydrogen peroxide and 3,3′-diaminobenzidine tetra hydrochloride (Sigma) for 3 min. Reaction was stopped by washing strips with water. Analysis was performed using a graphical method (Image version 1.44 software, National Institutes of Health, Bethesda, MD, USA) to generate a protein lane profile plot of each antigenic fraction, and then peaks referring to bands were compared with a protein standard marker (Real Biotech, RECOMTM Blue Wide Range Prestain Marker, Banqiao, Taiwan) to estimate their relative molecular weight.
2.7. Statistical analyses

Analyses were performed using the GraphPad software package v.5.0 (GraphPad Software, San Diego, CA, USA). Cut-off points were established using a 2-graph receiver operating characteristic curve (TG-ROC) (Greiner et al., 1995) based on ELISA results from positive patients (G1) and negative ones (G2 and G3). ELISA reactivity index (RI) was obtained by the ratio between OD and cut-off. Values of RI greater than the optimum point of reaction for each extract were considered positive (RI > 1). Sensitivity (Se) and specificity (Sp) were calculated according to the following formulas: Se = a/(a + b) and Sp = d/(c + d), where a = true positive, b = false positive, c = false negative, and d = true negative (Youden, 1950).

Receiver operating characteristic curves (ROC) were built to describe the tests (Martinez et al., 2003). The area under the ROC curve (AUC), an overall index of diagnostic accuracy, was calculated; values close to 1 indicate an informative test and close to 0.5 indicate an uninformative test (Hanley and McNeil, 1982). AUC comparison between antigenic preparations was made using the method described by Hanley and McNeil (1983), considering P < 0.05. Likelihood ratio (LR), an efficiency diagnostic parameter (Greiner et al., 1995), was calculated, as follows: LR+ = Se/(1 − Sp), which indicates how likely patients with NCC are to have a positive test result compared with other patients, and LR− = (1 − Se)/Sp, defined as the probability of having a negative test result for patients with NCC. P values of <0.0001 were regarded as significant, and 95% confidence intervals (CI) were provided for Se, Sp, and AUC.

3. Results

3.1. ELISA using SE and T. saginata metacestode antigenic fractions

Fig. 1 shows the absorbance values obtained by ELISA using SE, CM S1, CM S2, DEAE S1, and DEAE S2. The positivity rates of the group with NCC diagnosed (G1, n = 45) were as follows: 88% (40/45) for SE, 86% (39/45) for CM S1, 88% (40/45) for CM S2, and 84% (38/45) for DEAE S1. The DEAE S2 fraction had the highest positivity rate of 93% (42/45). In the group of patients infected with other parasites (G2, n = 50), 20% (10/50) of the samples were positive for SE and 16% (8/50) for CM S1. When testing DEAE S1 and DEAE S2, the positivity rate was 10% (5/50). The CM S2 fraction had the lowest positivity rate: 6% (3/50). Regarding the healthy group (G3, n = 45), the positivity rate was 4% (2/45) for SE, CM S1, DEAE S1, and DEAE S2, and 6% (3/45) for CM S2.

Cross-reactivity at G2 (RI > 1) was observed when testing serum samples from patients infected with hookworm only (1/5 SE, CM S1, CM S2, DEAE S1, and DEAE S2), S. mansoni (2/4 SE and CM S1, and 1/4 DEAE S1 and DEAE S2), S. stercoralis (2/7 SE and 1/7 CM S1), A. lumbricoides (1/6 SE, CM S2, and DEAE S1), T. solium (1/6 SE, CM S1, CM S2, DEAE S1, and DEAE S2). Cross-reactivity was also observed in the test of co-infected patients: hookworm + A. lumbricoides (1/1 SE, CM S1, CM S2, DEAE S1, and DEAE S2), E. histolytica + G. lamblia (1/1 SE and CM S1), and H. nana + E. vermicularis (1/1 SE, CM S1, and DEAE S2).

Results of sensitivity and specificity according to the cut-off point established by TG-ROC were 88.8% and 87.3% for SE, 86.6% and 89.4% for CM S1, and 84.4% and 92.6% for DEAE S1, respectively. The CM S2 and DEAE S2 fractions showed the best diagnostic performance with the highest values for sensitivity and specificity: CM S2, 88.8% and 93.7%; DEAE S2, 93.4% and 92.6%, respectively. Comparing the ROC curves, DEAE S2 was the most efficient fraction to distinguish patients with NCC (G1) from control groups (G2 and G3) according to the AUC value (0.987). When considering AUC values obtained from SE (0.951; P = 0.0178), CM S1 (0.953; P = 0.0005), CM S2 (0.965; P = 0.0005), and DEAE S1 (0.968; P = 0.0287), a lower efficiency was observed. The LR+ values for SE (LR+ = 7.04) and CM S1 (LR+ = 8.23) indicated a moderate probability of a true-positive NCC case. However, values of LR+ for CM S2 (LR+ = 11.46), DEAE S1 (LR+ = 12.67) pointed to an efficient test. The LR− values for SE (LR− = 0.11), CM S1 (LR− = 0.14), CM S2 (LR− = 0.11), and DEAE S1 (LR− = 0.16) indicate that tests had a moderate...
effect on diminishing the probability of disease, while a low LR− (<0.1) for DEAE S2 (LR− = 0.07) practically excludes the chance of a patient to be infected (Fig. 2).

3.2. Immunodominant protein recognition

Immunoblotting was performed with all ELISA reactive serum samples. In G1, there was recognition of immunodominant proteins (12 to 14, 24, 39 to 42, 47 to 52, 64 to 68, 70, 75, 80, 86, 100, and >140 kDa) for SE. High-molecular-weight bands (≥80 kDa) were eliminated for NCC diagnosis in agreement with Barcelos et al. (2007). A higher frequency of proteins from 39 to 42, 47 to 52, and 64 to 68 kDa was detected in patients who had active NCC, when using a DEAE S2 fraction (Fig. 3A). Patients who had inactive NCC manifested a higher frequency of the proteins with high molecular weight using SE and the 4 antigenic fractions (Fig. 3B). Samples from G2 reacted with high-molecular-weight bands (≥80 kDa), when using SE, CM S1, DEAE S1, and DEAE S2 fractions. No bands were recognized by samples from patients of G3.

4. Discussion

*T. saginata* metacestode antigen is an alternative to the *T. solium* metacestode antigen demonstrating high sensitivity and specificity (Oliveira et al., 2007). Although heterologous antigen from *T. saginata* metacestodes has been used with satisfactory results, research studies have been conducted to test its purification with the purpose of selecting antigens present in this helminth that are able to react with antibodies induced by *T. solium*-infected individuals, increasing the positive reactions and minimizing the cross-reactivity (Gonçalves et al., 2010; Nunes et al., 2010; Oliveira et al., 2010; Ribeiro et al., 2010).

Fig. 2. Receiver operating characteristic curves (ROC) indicating optimum point of reaction (cut-off), sensitivity (Se), specificity (Sp), area under curve (AUC), and likelihood ratios (LR+ and LR−) for detection of IgG antibodies to *T. solium* metacestodes in serum samples using SE of *T. saginata* metacestodes, CM S1 and CM S2 fractions, and DEAE S1 and DEAE S2 fractions.
Ion-exchange chromatography separates proteins based on molecular charge, so proteins are bound by reversible electrostatic interactions. Negatively charged support (CM) will bind a compound with an overall positive charge. Conversely, a positively charged support (DEAE) will bind a compound with an overall negative charge. The present study was the first attempt using ion-exchange chromatography to obtain fractions of T. saginata metacestodes potentially applicable in the immunodiagnosis of human NCC. Purification of antigens is an available tool to obtain a simple, specific, sensitive, and low-cost immunodiagnostic test for NCC detection.

The TG-ROC curve analysis provided an optimal cut-off point for each antigenic fraction studied, so a different IgG detection profile for each fraction tested was observed. CM S2 fraction showed greater specificity and DEAE S2 fraction greater sensitivity. Values of LR+ greater than 10 confirm the diagnosis as proposed by Jaeschke et al. (1994), and those of LR− below 0.1 exclude diagnosis. As a result, LR values showed that DEAE S2 fraction had a remarkable diagnostic performance to detect IgG in patients with NCC, reaching values of LR+ = 12.67 and LR− = 0.07.

Using antigen from T. saginata metacestodes, Oliveira et al. (2007) found proteins of 39 to 42 and 64 to 68 kDa, when testing serum samples of patients with active NCC. Barcelos et al. (2007) demonstrated that proteins of 47 to 52, 64 to 68, and 70 kDa are specific markers when using serum and/or cerebrospinal fluid samples of patients with active NCC. A fraction obtained for ion-exchange chromatography of Taenia hydatigena metacestode was evaluated for use in the immunodiagnosis of ovine cysticercosis, revealing the presence of 68-kDa protein (Panda et al., 2000). The DEAE S2 fraction revealed higher frequency of the proteins of 39 to 42, 47 to 52, and 64 to 68 kDa, in patients with active NCC. It is suggested that bands described above are equivalent to the ones found herein, and the DEAE S2 partially purified fraction should be relevant in the immunodiagnosis of human NCC. In our study, serum samples from patients with inactive NCC recognized the band of 75 kDa when using the SE and CM S2 and DEAE S2 fractions, suggesting that this band is a specific marker of inactive NCC.

Previous studies that employed heterologous antigens from T. saginata metacestodes and/or homologous antigens from T. solium metacestodes showed reactivity with high-molecular-weight bands (≥80 kDa) (Barcelos et al., 2007). The loss of specificity for the DEAE S2 fraction was mainly due to reactivity with components presenting high molecular weight in G2. In subsequent studies, the fractionation of antigens from T. saginata metacestodes using gel filtration chromatography will allow the exclusion of high-molecular-weight components, increasing the specificity of immunodiagnostic tests. In patients with other parasitic infections, there was no cross-reactivity when the CM S2 fraction was used in immunoblotting. An advantage of our test is the use of a heterologous antigen that leads to comparable results with the homologous antigen. Considering the results achieved here, DEAE S2 and CM S2 fractions have been used for the first time as an antigen in ELISA and immunoblotting for NCC immunodiagnosis. T. saginata metacestode antigenic fractions demonstrated high sensitivity and specificity when applied to the sera of individuals with NCC or other parasitic infections or of healthy individuals. T. saginata metacestode antigenic fractions obtained by ion-exchange chromatography showed high diagnostic parameters,

![Fig. 3. Immunoblotting for detection of IgG antibodies to T. solium metacestodes in serum samples using SE of T. saginata metacestodes, CM S1 and CM S2 fractions, and DEAE S1 and DEAE S2 fractions from patients with active NCC (A) and inactive NCC (B). Peaks of intensity, indicative of the bands detected, were generated by ImageJ version 1.44. MW = Molecular weight standard in kilodaltons (kDa).](image-url)
suggesting sources of immunodominant polypeptides. In conclusion, our data demonstrate that CM S2 and DEAE S2 fractions, obtained from \textit{T. saginata} metacestodes, fractionated by ion-exchange chromatography are an important source of immunodominant polypeptides potentially applicable in NCC immunodiagnosis.

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