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Recombinant expression of *Taenia solium* TS14 antigen and its utilization for immunodiagnosis of neurocysticercosis

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Abstract

In order to evaluate the potential use of TS14 antigen in an enzyme-linked immunosorbent assay (ELISA) for immunodiagnosis of neurocysticercosis (NC), its open reading frame (ORF) was amplified by RT-PCR from mRNA isolated from *Taenia solium* cysticerci. The ORF was subcloned into the expression vector pET-28a, and was used to transform *Escherichia coli* BL21 (DE3) cells to produce TS14 antigen. The His-tagged expressed protein was purified on a nickel affinity column. Using the $_{HIS}$TS14 as antigen, ELISA was positive for 100% of cerebrospinal fluid (CSF) and 97% of serum samples from NC patients. No positive results were observed with sera and CSF samples from control groups. Cross-reactivity with sera from patients with schistosomiasis and Chagas' disease was not observed. Serum samples from patients with taeniasis were evaluated and 2 of 13 cases showed reactivity in this assay. Our data indicate the usefulness of $_{HIS}$TS14 in ELISA for an accurate and rapid assay for diagnosis of NC and seroepidemiological studies.
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Keywords: *Taenia solium*; Neurocysticercosis; TS14; Diagnosis; Recombinant antigen; Enzyme-linked immunosorbent assay

1. Introduction

Taeniasis and cysticercosis are caused by the cestode *Taenia solium*, which is considered a public health problem in many developing countries where social, economic and cultural conditions favor the maintenance of this zoonosis (Flisser, 1994). Neurocysticercosis resulting from *T. solium* larvae developing in the central nervous system is currently recognized as the main cause

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of acquired epilepsy throughout the world (Commission on Tropical Diseases, 1994), affecting human health and productivity. The disease is the major source of neurological morbidity in developing countries (Garcia, 2002). It is estimated that 400,000 people in Latin America have this symptomatic disease (Bern et al., 1999). Cysticercosis is endemic in several areas of Brazil, and is most likely the major cause of epilepsy (Trevisol-Bittencourt et al., 1998). Few studies on neurocysticercosis in Brazil have been published but the gravity of the problem was demonstrated in some areas (Agapejev, 1996, 2003; Gomes et al., 2002). Diagnosis is based on the combination of clinical and epidemiological criteria and on neuroimaging and immunological methods. These are not always available in areas where the disease is endemic.

The enzyme-linked immunosorbent assay (ELISA) has been used to detect antibodies in NC patients in both cerebrospinal fluid (CSF) and serum samples (Ev et al., 1999; Bueno et al., 2000; Peralta et al., 2002). However, the availability of suitable and standardized antigens in large amounts from *T. solium* is a prerequisite. In addition, cross-reactivity between antigens from cysticerci and antibodies present in sera from patients with other parasitosis has been observed (Bueno et al., 2000; Bragazza et al., 2002; Ishida et al., 2003). Extensive studies with glycoproteins purified from the metacestode stage of *T. solium* have indicated that these molecules are appropriate for immunodiagnosis of NC by enzyme-linked immunoelectrotransfer blot (EITB) (Tsang et al., 1989; Rodrigues-Canul et al., 1997). However, the use of this test is limited due to the assay technology, high cost and complexity of the antigen purification process.

The use of recombinant DNA technology permits the obtainment of high amounts of purified proteins, enabling the development of more sensitive and specific assays. Among the analyzed antigens, the 14 kDa protein isolated from *T. solium* cysticerci (TS14) is considered one of the most promising (Greene et al., 2000; Obregon-Henao et al., 2001). The objective of this study was to produce recombinant TS14 antigen in *Escherichia coli* and to evaluate its potential use as an antigen for immunodiagnosis of NC in endemic areas.

2. Materials and methods

2.1. Serum and CSF samples

Samples were obtained from patients presenting at the Faculty of Medicine Hospital of the University of Sao Paulo in Brazil. Forty-one CSF and 30 serum samples from patients with NC were collected. The diagnosis was confirmed by an imaging exam and clinical manifestations as well as biochemical-cytological and immunological tests for the detection of antibodies (Bueno et al., 2000). Imaging classification (Machado et al., 1990) was used, allowing the identification of different types: 30 CSF samples from patients with degenerating cysts (single cyst in one case and multiple cysts in 29 cases), 1 with calcified cysts (two cysts) and 10 CSF samples for which no neuroimaging information was available. Regarding serum samples, 8 were obtained from patients with intact cysts (single in two cases and multiple in 6 cases), 19 had degenerating cysts (single in 3 cases and multiple in 16 cases) and 3 harbored calcified cysts (single in 2 cases and multiple in 1 case).

The control group consisted of 36 serum samples from apparently healthy individuals originating from non-endemic areas for cysticercosis and 29 CSF samples obtained from patients with other neurological disorders. None of these CSF samples exhibited laboratory alterations and the diagnosis of meningitis was excluded. All CSF and serum samples from the control group were determined to be non-reactive by immunological tests, ELISA and immunoblot using *T. crassiceps* and *T. solium* antigens (Bueno et al., 2000).

Sera from patients with other parasitosis were used to assess the specificity of the assay, including 9 with schistosomiasis confirmed by clinical and laboratory (Kato-Katz technique) examinations and 14 with Chagas' disease confirmed by hemoculture. Thirteen serum samples from patients from São João da Ponte (State of Minas Gerais, Brazil) with taeniasis, as confirmed by stool examinations for the detection of *Taenia* eggs, were also available. These patients presented a negative clinical laboratory diagnosis for NC and were also negative for anti-cysticercus antibodies (Bueno et al., 2000).

The Ethics Committee for the Analysis of Research Projects of the FCF/USP approved present study (approval 188/2003), which complied with Resolution No. 196/96 of the National Health Council of the Brazilian Ministry of Health.

2.2. Native antigens

T. solium cysticerci were obtained from the muscles of a naturally infected pig from São João da Ponte (state of Minas Gerais, Brazil). Native antigens from *T. solium* cysticercis were obtained for immunoblot analysis. Vesicular fluid was obtained by aspiration from intact cysticerci with a syringe coupled with a

13 by 0.4 mm needle, as described by Espíndola et al. (2005). Membrane and scolex were separated by dissecting the cysticerci with a scalpel and were homogenized in phosphate-buffered saline (PBS; 0.01 M, pH 7.2, 0.0075 M Na_2HPO_4, 0.025 M NaH_2PO_4, 0.15 M NaCl), as reported by Pinto et al. (2000). The three antigens (vesicular fluid, membrane and scolex) were centrifuged at 15,000 × *g* for 60 min at 4 °C. The supernatants were sonicated (at 20 kHz and 1 mA for four 30-s periods in an ice bath) and then centrifuged at 15,000 × *g* for 60 min at 4 °C, and the supernatants were stored.

2.3. TS14 recombinant antigen

2.3.1. Parasite RNA and cDNA

RNA was isolated from *T. solium* cysticerci using the Total RNA Isolation System (Promega, Madison, WI, USA). The TS14 open reading frame was obtained by reverse transcription, RT-PCR (Ready-to-go kit, Amersham Biosciences, Uppsala, Sweden), as described by the manufacturer. The PCR primers used were synthesized (Invitrogen do Brasil, Sao Paulo, SP) according to the previously published sequence (GenBank accession number AF082829): 5′GCCATGGCGCATATGCGTGCCTACATTGTGCTT3′ and 5′CCGGATCCTTAAGCAGTTTTTTTCTTAGGACC3′, which contained *Nde*I and *Bam*HI restriction sites to facilitate cloning steps. PCR cycling conditions were as follows: one cycle at 42 °C for 30 s followed by 35 cycles at 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min.

The PCR product was purified from 1% agarose gel (Concert Rapid Gel Extraction System – Life Technologies), cut with *Nde*I and *Bam*HI and subcloned into the expression vector pET28a (Novagen, Madison, WI), previously cleaved with the same enzymes. The recombinant plasmid containing the TS14 ORF was dubbed pET28TS14.

2.3.2. Expression and purification of $_{HIS}$TS14

The plasmid pET28TS14 was used to transform *E. coli* BL21 (DE3) (Stratagene, La Jolla, CA) calcium chloride competent cells (Sambrook et al., 1989). Transformed cells were grown in LB broth medium with 25 μg/mL kanamycin for 12 h, diluted 1:100 in LB plus kanamycin and expression was induced at an Absorbance (6 0 0) = 0.6 by the addition of isopropylthio-β-D-Galactoside (IPTG) to 0.4 mM for 4 h at 30 °C. Cells were harvested by centrifugation at 3000 × *g* for 20 min at 4 °C, and the pellet was washed with 50 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 300 mM NaCl (column buffer) and afterwards centrifuged at 3000 × *g* for 20 min. For cell disruption, the pellet was solubilized in the column buffer containing 0.25% sodium-*N*-lauroylsarcosine (Amersham Biosciences) with gentle agitation for 20 min, and the suspension was centrifuged at 12,000 × *g* for 15 min. The supernatant was separated on a Ni-NTA column (Qiagen, Valencia, CA), according to manufacturer instructions, and the $_{HIS}$TS14 was eluted with an increased concentration of imidazole. The protein concentration was determined by BioRad-Protein Assay Reagent (Bio-Rad, Laboratories Inc., Hercules, CA).

2.4. Monoclonal antibody (MoAb) against $_{HIS}$TS14

Balb/c mice were immunized with 20 μg purified $_{HIS}$TS14 to generate MoAbs, as described by Espíndola et al. (2000). The Ethics Committee for Experimental Animals of the FCF/USP approved the animal manipulation (project 13/2003) adopted by the Brazilian Committee for Experimental Animals (COBEA).

2.5. SDS-PAGE, Tricine-PAGE and immunoblot analysis

The $_{HIS}$TS14 expression and purification was analyzed by Tricine-PAGE (Schagger and Von Jagow, 1987) under reducing conditions and coomassie blue staining.

For the immunoblot, the purified $_{HIS}$TS14 and native antigens were resolved on 15% SDS-PAGE gel (1 μg/mm), as described by Laemmli (1970), under reducing and non-reducing conditions (native antigens) and then blotted onto a Polyvinylidene Difluoride membrane (Millipore Corp., Bedford, MA). Membranes were cut into 3-mm-wide strips, blocked for 2 h with 5% skimmed milk (Molico, Nestlé, Sao Paulo, Brazil) containing 0.05% Tween 20 (PBS-T), washed in PBS-T, and then incubated for 18 h at 4 °C with serum samples diluted 1:50, CSF samples 1:4, and anti-TS14 MoAb 1:1000, in 1% skimmed milk prepared in PBS-T. After further washing, strips were incubated for 1 h with Alkaline phophatase-labeled anti-mouse IgG conjugate (Bio-Rad) and Alkaline phophatase-labeled anti-human IgG conjugate (Sigma, St. Louis, MO), diluted 1:2000 and 1:1500, respectively, and prepared in 1% skimmed milk in PBS-T. After additional washing, the antigen–antibody complexes were developed by incubation with 0.01% 5-bromo-4-chloro-3-indolyl-phosphate pre-dissolved in *N*,*N*-dimethylformamide (DMF) (Sigma) and 0.02% nitro blue tetrazolium (Sigma) pre-dissolved in 70% DMF, and then diluted in 0.01 M $NaHCO_3$ and 0.001 M $MgCl_2$.

2.6. ELISA

The ELISA was performed according to Pinto et al. (2000). The 96 well-plates (Costar, Corning, USA) were sensitized with 100 μL/well of $_{HIS}$TS14 antigen (1 μg/well) diluted in 0.5 M carbonate–bicarbonate buffer (pH 9.6) for 14 h in a humidified chamber at 4 °C. Wells were blocked for 1 h with 5% skimmed milk in PBS-T, and then incubated for one hour with serum or CSF samples diluted 1:100 and 1:10, respectively. Anti-human IgG peroxidase-conjugate (Amersham Biosciences) was diluted 1:2000 for serum and 1:1500 for CSF, and plates were incubated for 1 h. After each incubation step, the plates were washed using an automatic washer, with five cycles of PBS-T. The enzymatic reaction was developed with the chromogenic substrate tetramethylbenzidine H_2O_2 (Sigma) in the dark for 15 min and stopped with 2N H_2SO_4. Absorbance values were read from a plate spectrophotometer (Multiskan-Labsystems) at 450 nm. The samples and conjugate were diluted in 1% skimmed milk prepared in PBS-T and all incubations were carried out at 37 °C. Each unknown sample was tested in duplicate and mean absorbance was determined and compared with the cut-off value. A blank (assay without sample) was also included in each plate. The assay was monitored by including standards in each plate, chosen among the positive and negative samples. The cut-off was calculated from the mean of the absorbance values of the control group (negative) plus 2 standard deviations.

3. Results

The 258 nt TS14 open reading frame, which encodes for a protein of 86 amino acids was obtained by RT-PCR from *T. solium* cysticerci total RNA, and subcloned into the pET28a expression vector. After cell disruption in the presence of 0.25% sodium-*N*-lauroylsarcosine, the protein was efficiently purified on a Ni-NTA affinity column in the fraction containing 100 mM imidazol. The recombinant protein possesses at its N-terminus, 20 additional amino acids encoded by pET28a, including the HIS-tag. Thirty mg of purified $_{HIS}$TS14 were obtained per liter of *E. coli* culture.

As predicted, the observed molecular mass of recombinant $_{HIS}$TS14 was approximately 11.7 kDa on Tricine-PAGE analysis (Fig. 1A). By immunoblot, $_{HIS}$TS14 was recognized by antibodies in serum and

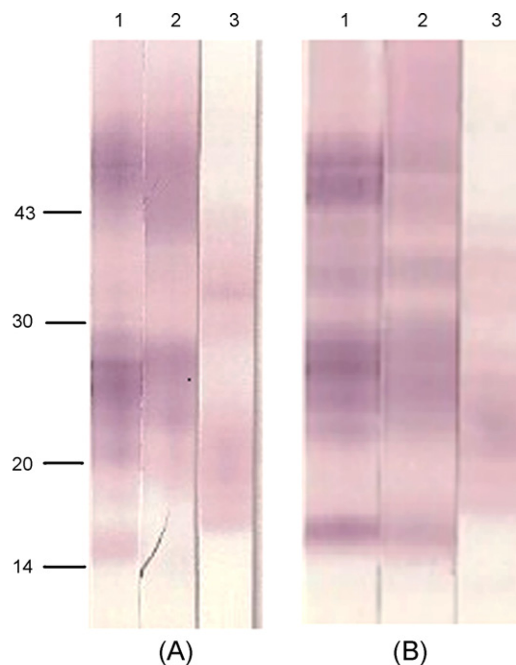

Fig. 2. Immunoblot analysis of native antigens from *Taenia solium* cysticerci: (1) vesicular fluid; (2) membrane; (3) scolex probed with MoAb against $_{HIS}$TS14 under non-reducing (A) and reducing conditions (B).

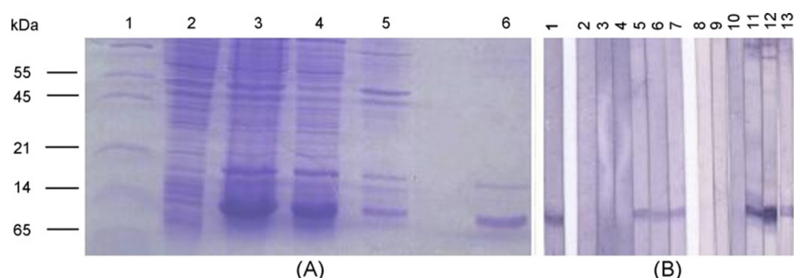

Fig. 1. (A) Trycine-PAGE analysis of $_{HIS}$TS14 purification. Lane 1, molecular-weight markers; lane 2, total *Escherichia coli* proteins from uninduced cultures containing the recombinant plasmid; lane 3, post-induction cell proteins of bacteria containing the recombinant plasmid; lane 4, supernatant after solubilization in 0.25% sodium-*N*-lauroylsarcosine; lane 5, proteins unbound to Ni-NTA column; lane 6, elution fraction with 100 mM imidazole. (B) Immunoblot analysis of TS14 antigen probed with: (1) MoAb against $_{HIS}$TS14; (2–4) serum sample from control group; (5–7) serum sample from NC patients; (8–10) CSF sample from control group; (11–13) CSF sample from NC patients.

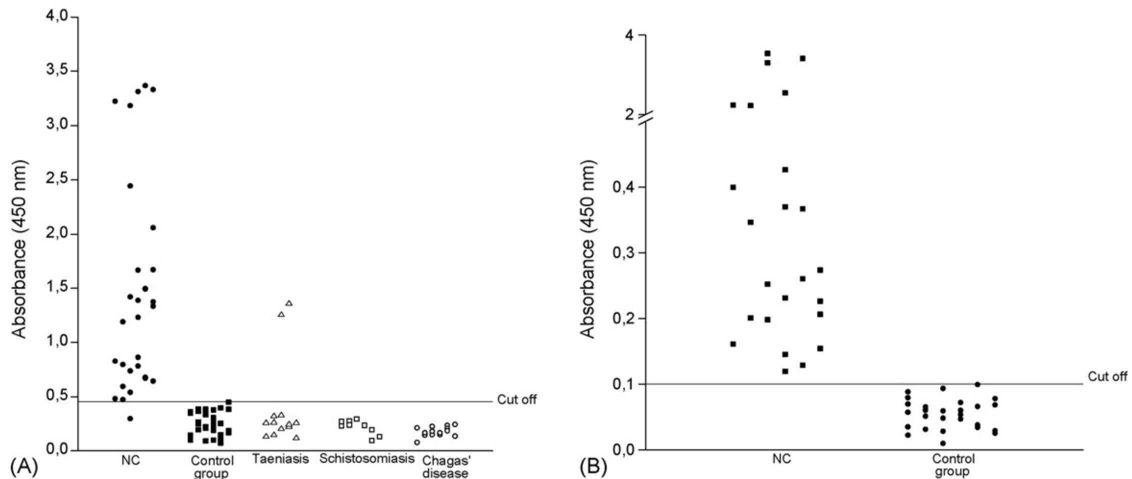

Fig. 3. Results of ELISA using $_{HIS}$TS14 antigen. In (A) serum samples of NC patients, control group, taeniasis, schistosomiasis and Chagas' disease; in (B) CSF samples of NC patients and control group.

CSF samples from patients with NC (Fig. 1B) and not by samples from the control group. Furthermore, MoAb directed against $_{HIS}$TS14 was tested with native antigens from scolex, membrane and vesicular fluid antigens and showed reactivity with the bands between 14 and 60 kDa in the 3 fractions of cysticerci, but more intensively with vesicular fluid under reducing condition (Fig. 2B). Under non-reducing conditions, MoAb anti-$_{HIS}$TS14 reacted with two groups of antigens in molecular weight ranging from 20 to 28 kDa and 40 to 60 kDa (Fig. 2A).

To assess the diagnostic value of $_{HIS}$TS14, we further tested its immunoreactivity by ELISA. A positive reaction to $_{HIS}$TS14 was observed in 97% of sera from NC patients (30 cases) based on a cut-off value of 0.454. Only one NC patient serum (multiple cysts in degeneration) was found negative in ELISA. No positive results were observed with sera from the control group (36 cases), or sera from patients with schistosomiasis (9 cases) and Chagas' disease (14 cases), and 2 of 13 cases of taeniasis were positive in ELISA (Fig. 3A). ELISA results with $_{HIS}$TS14 and CSF samples showed that all 41 patients (100%) with NC were positive, demonstrating high sensitivity based on a cut-off value of 0.100. ELISA performed with CSF samples from the control group (29 cases) showed no positive reactions (Fig. 3B).

4. Discussion

Native TS14 has proven to be useful for diagnosis of NC using EITB (Tsang et al., 1989; Michault et al., 1990; Greene et al., 1999) and ELISA (Pardini et al., 2001). Therefore, we decided to use the recombinant DNA technology to produce TS14 recombinant antigen and evaluate its potential use as an antigen for NC immunodiagnosis. The TS14 antigen was efficiently expressed in *E. coli*, as a fusion with HIS-tag, and purified by only one step of purification on the Ni-NTA affinity column and used to generate MoAb.

MoAb anti-$_{HIS}$TS14 reacted with antigens in molecular weight ranging from 14 to 60 kDa from scolex, membrane and vesicular fluid from *T. solium* cysticerci, showing more intensity in vesicular fluid, as displayed in the immunoblot under reduced conditions. Antigens under 20 kDa were not detected under non-reducing conditions. Similarly, Restrepo et al. (2000) described that *Lens culinaris* binding glycoproteins from *C. Cellulosae* migrated as two broad groups of bands in the 24–30 kDa and 42–55 kDa ranges under non-reducing conditions, and five bands of 12–28 kDa were detected under reducing conditions. Previous experiments have suggested that the presence of a cysteine residue within the TS14 sequence may be implicated in dimer formation via disulfide bond (Greene et al., 2000; Sako et al., 2000), and these subunits are components of larger antigens (Greene et al., 1999; Plancarte et al., 1999). Hancock et al. (2003) reported that the diagnostic antigens at 14, 18 and 21 kDa, as well as some larger disulfide bonded antigens, are all members of a very closely related family of proteins, the 8 kDa antigens, and possibly accumulate in cyst fluid.

The TS14 recombinant antigen was only identified by antibodies in serum and CSF samples from NC patients, and not by control groups in immunoblotting, demonstrating that the test has potential for the immunodiagnosis of NC. No correlation was observed between the evolutionary phases of NC and reactivity of ELISA.

Only one false negative result occurred in a serum sample from a patient with multiple cysts in degeneration. This negative result may be explained in part by the formation of immunocomplexes in the presence of excess antigens (Peralta et al., 2002). None of the sera from the control group recognized the $_{HIS}$TS14 antigen as well as sera from patients with schistosomiasis and Chagas' disease.

Thirteen sera samples from taeniasis patients were assayed by ELISA, two of which were positive. We cannot exclude the possibility that these taeniasis patients had cysticercosis, since the contamination of *T. solium* eggs can occur by auto-infection in taeniasis individuals or by hetero-infection through contaminated food and water (Trevisol-Bittencourt et al., 1998; Takanayagui and Leite, 2001; Garcia et al., 2003; Hawk et al., 2005). The most difficult parameters in the immunodiagnosis of NC have been attributed to the cross-reactivity between antigens from cysticerci and antibodies in sera from patients with taeniasis due to *T. solium* or *T. saginata*, or hydatidosis, as observed by Ev et al. (1999). However, Tsang et al. (1989) and Michault et al. (1990) reported that 14 kDa antigen proved to be specific to the immunodiagnosis of NC, and no cross-reactivity was observed with sera from patients with taeniasis or other parasitosis.

Our results indicate that the absence of post-translational modifications did not impair the antigenicity and specificity for recombinant TS14, despite some studies having reported poor antigenicity for antigens lacking glycosylation (Obregon-Henao et al., 2001). No significant difference between the sensitivity and specificity of the test was found for serum or CSF. Nevertheless, the use of serum samples for the immunodiagnosis of neurocysticercosis has a number of advantages: serum can be obtained in a less invasive manner than CSF and seroepidemiological studies can map endemic areas (Bueno et al., 2000; Del Brutto et al., 2001).

The immunological method of choice for NC diagnosis adopted by the Pan American Health Organization is the EITB uses a partially purified antigen (Tsang et al., 1989). The EITB for NC is 100% specific and 98% sensitive for patients with two or more cysts. However, this assay needs parasite material, which is costly and technically complicated. The TS14 antigen, chemically synthesized, has been successfully used for immunodiagnosis of NC (Greene et al., 2000; Hancock et al., 2003; Scheel et al., 2005). The recombinant TS14 together with ELISA technology is a less expensive and simpler diagnosis method. Our data show that the utilization of TS14 for the detection of anti-cysticercus antibodies in CSF and serum samples using ELISA was efficient, with high sensitivity and specificity. Our data suggest the use of recombinant TS14 antigen in seroepidemiological studies for evaluating the real situation of NC in Brazil and other countries at reduced cost. Further analysis with a large number of serum samples from different areas should validate the diagnostic efficiency of the TS14.

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