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Taenia solium: A two-dimensional Western blotting method combined with the use of an EST-library for the identification of immunogenic proteins recognized by sera from neurocysticercosis patients

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ABSTRACT

Commercial antigens used to diagnose human neurocysticercosis (NCC) are obtained from either a soluble parasite extract or a parasite-derived glycoprotein fraction. The aim of the present study was to identify antigenic proteins as potential diagnostic candidates in this context. Soluble immunogenic proteins from *Taenia solium* cysticerci were identified by two-dimensional electrophoresis Western blotting using human sera from Nicaragua confirmed to be positive for NCC by computer tomography. Six antigenic proteins were identified and sequenced by liquid chromatography–mass spectrometry. Among these immunogenic proteins, a novel sequence was found and named Tsol-p27. To determine the antigenicity of Tsol-p27, the previously reported antigen TsolHSP36 and the new Tsol-p27 were expressed as recombinant proteins and evaluated serologically. Immunoblotting demonstrated that Tsol-p27 was recognized by sera from 13 NCC-positive humans, whereas TsolHSP36 was identified by only two of those 13 positive sera. None of the antigens were recognized by negative control sera. Despite the limited number of serum samples evaluated in this study, the results indicate that Tsol-p27 might be a suitable candidate for diagnosis of human NCC.

1. Introduction

Taenia solium cysticercosis represents an important health problem in many Asian, African, and Latin American countries with poor sanitary conditions, and the same is now being seen in some high-income nations as the result of travel to or immigration from endemic areas (Mahanty and Garcia, 2010; Sciutto et al., 2000). Humans are accidentally infected with *T. solium* when they ingest embryonated eggs passed in feces from the intestine of a host harboring the adult stage of the tapeworm (Flisser, 1994; Garcia et al., 2005a,b). Once inside a human, the embryos hatch and penetrate the intestinal wall, and then disseminate into several body tissues, where they enlarge and mature into cysticerci (Garcia et al., 2002). The main clinical manifestation of the infection is neurocysticercosis (NCC), which occurs when cysticerci are established in the brain (Garcia et al., 2005a,b). The clinical signs and symptoms of NCC are inconsistent and nonspecific, and include severe headache, hydrocephalus, blindness, epilepsy, and various neurological symptoms (Del Brutto, 2005). In endemic countries, this disease has been identified as the major etiological agent of epileptic seizures, accounting for 30–50% of all cases of late-onset epilepsy (Lescano et al., 2009).

The methods used to diagnose human NCC include neuroimaging tests such as magnetic resonance imaging (MRI) and computed tomography (CT) (Carpio et al., 1998). Neuroimaging techniques are expensive and are not often available to larger proportions of the populations in endemic regions (Diaz et al., 1992). Other diagnostic tools include histological demonstration of parasites in biopsies and cerebrospinal fluid (CSF), and immunological methods. Among the latter, an enzyme-linked immunoelectrotransfer blot (EITB) assay has proven to be a sensitive and specific technique (Tsang et al., 1989). This immunoassay depends on crude material from the parasite, and it requires special expertise to prepare antigens and perform the test (Deckers and Dorny, 2010; Dorny et al., 2003). Therefore, efforts have been focused on developing improved diagnostic tools that offer sensitivity, specificity, and suitability for seroepidemiological studies, all at a low cost (Dorny et al., 2003; Ito and Craig, 2003). Recombinant antigen production is an option that can eliminate the need for crude parasite material and facilitate diagnosis of the disease. Here, we report six immuno-

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reactive proteins from *T. solium* cysticerci identified by two-dimensional electrophoresis (2-DE) Western blot analysis using sera from NCC patients in Nicaragua. We selected and produced two of these as recombinant proteins in *Escherichia coli*, namely, a novel sequence we named Tsol-p27 and the previously described TsolHSP36 (Ferrer et al., 2005).

2. Materials and methods

2.1. Parasite material and serum samples

T. solium cysticerci were removed from the muscles of naturally infected pigs obtained at abattoirs in León, Nicaragua, and then washed in phosphate-buffered saline (PBS pH 7.5). Two groups of human serum samples were used to identify the native proteins and evaluate the recombinant antigens that were produced. The positive control group consisted of four patients with NCC confirmed by CT and ELISA (Cypress) and nine individuals with positive serology by ELISA (Cypress). The healthy control group comprised 13 apparently healthy humans; all 26 subjects lived in León, Nicaragua. Human serum samples and cysticerci were obtained at the National Autonomous University of Nicaragua–León (UNAN–León) and stored at −20 °C until used. The study protocol was approved by the ethics committee of the Faculty of Medical Science at UNAN–León.

2.2. Two-dimensional electrophoresis (2-DE)

Three cysticerci were disrupted in 500 μl of PBS by mechanical pressure and homogenized with a protease inhibitor cocktail (Invitrogen). The extracted proteins (30 μg) were mixed with rehydration buffer containing 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.4% (w/v) DTT, and 0.002% (w/v) bromophenol blue. Isoelectric focusing (IEF) was conducted using a Multiphor system (Pharmacia Biotech) and IPG strips with a linear pH range of 4–10 or 4–7 (GE Healthcare) at a step voltage of 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. The strips from IEF were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gel in the second dimension, according to the manufacturer's instructions (Pharmacia Biotech). Samples were analyzed in triplicate to assess the reproducibility of protein patterns. The gels were either stained with Coomassie Brilliant Blue (Bio Rad) or blotted onto nitrocellulose membranes (GE Healthcare).

2.3. Western blot analysis

The proteins separated by 2-DE and the Tsol-p27 and TsolHSP36 recombinant antigens were transferred onto nitrocellulose membranes (GE Healthcare). The membranes were blocked for 2 h with 5% skim milk in PBS and then rinsed in washing buffer containing PBS and 0.05% Tween 20. Thereafter, the membranes were incubated first for 3 h with human sera diluted 1:500 in 5% skim milk-PBS, and then for 1 h with rabbit anti-human IgG conjugated with peroxidase (Sigma) diluted 1:3000 in 5% skim milk-PBS. The subsequent washing steps and detection procedures were performed according to the ECL plus manual (GE Healthcare).

2.4. Sequencing of immunoreactive proteins

Spots recognized by positive human sera in the 2-DE Western blot and identified in the 2-DE gel stained with Coomassie Brilliant Blue (Bio Rad) were manually cut out and stored at −20 °C until used. Native proteins were trypsinized and 5–10 fragments were identified by liquid chromatography–mass spectrometry analysis (LC–MSMS) at the Protein Analysis Center, Karolinska Institutet, Solna, Sweden.

2.5. Isolation of cDNA encoding Tsol-p27 and TsolHSP36

The amino acid sequences corresponding to Tsol-p27 were found by searching our previously constructed expressed sequence tag (EST) library for *T. solium* cysticercus (Lundstrom et al., 2010), and the sequence corresponding to TsolHSP36 has been described elsewhere (Ferrer et al., 2005). The longest open reading frame (ORF) of each protein was selected to design the primers. Amplified PCR products were used in further cloning.

2.6. DNA sequencing and analysis of Tsol-p27

The ESTs were translated *in silico* using Virtual Ribosome version 1.1, and they served as an *in silico* library of translated ESTs. Amino acid sequences of peptides derived from Tsol-p27 were identified, and the corresponding ESTs were isolated and sequenced. DNA sequencing was done using a MegaBace 1000 system (Amersham Biosciences). The sequencing analysis of Tsol-p27 was performed at the National Center for Biotechnology Information (NCBI) using BLAST search option with a cut of 10^{-5}.

2.7. Cloning, expression, and purification of Tsol-p27 and TsolHSP36

The selected cDNA used to produce the recombinant Tsol-p27 and TsolHSP36 proteins was amplified using specific primers. For Tsol-p27 primer pair T sol-p27F 5′-CTGGGATCCGACGTTACCA AGAGTTTCAATAG-3′ (sense)/T sol-p27R 5′-CCG CTC GAG CTA GTG GTG GTG GTG GTG GTG GGA TCC CAA CAT CAC-3′ (antisense) and TsolHSP36, TsolHSP36F 5′-CTG GGA TCC ATG TCC ATC TTT CCG ACT CGT-3′ (sense)/TsolHSP36R 5′-CGG GAA TTC TTT AAA AAG AGG CGC CTC CAC AAC C-3′ (antisense) were used. Maxima Hot Start PCR Master Mix (2X) (Fermentas) was used in all polymerase chain reactions (PCRs). The PCR conditions were as follows: one cycle at 96 °C for 5 min followed by 30 cycles at 96 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min, and 72 °C for 7 min. The PCR experiments were performed on a Thermo Hybrid system. The PCR products of the Tsol-p27 and TsolHSP36 genes were subcloned in Bam HI/Xho1 and Bam HI/Eco R1 sites of the expression plasmid vector pGEX-4T-1 (Amersham Pharmacia) downstream of glutathione *S*-transferase (GST). Recombinant GST-fusion proteins were expressed in *E. coli* BL21 (Invitrogen) after induction with 1 mM isopropyl β-thiogalactopyranoside (IPTG) (Sigma). The soluble recombinant proteins were purified using magnetic beads coated with glutathione and PreScission Protease according to the manufacturer's instructions (GE Healthcare). The recombinant proteins were analyzed by 10% SDS–PAGE and visualized by staining with Coomassie Brilliant Blue. Antigenicity was evaluated by Western blotting.

3. Results

3.1. Identification of immunoreactive proteins

Antigens from cysticerci were separated on 4–10 and 4–7 IPG strips. The proteins were recognized by the four sera obtained from patients with NCC, and these immunogenic antigens were found to have p*I* values between 4 and 7. Comparison of the 2-DE Western blot with the 2-DE gel stained with Coomassie Brilliant Blue allowed us to localize antigenic spots (Fig. 1). A total of seven spots were cut out from the 2-DE gel and subjected to LC–MSMS analysis, and the immunoreactive proteins that were identified are presented in Table 1. One of those was a novel *T. solium* protein that was named Tsol-p27 (Genbank ID: BankIt1444149 Tsol-p27