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.

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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, hydro-cephalus, 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

JF751062). Serum samples from the healthy human control group did not give rise to any conflicting spots in 2-DE Western blotting (Fig. 1).
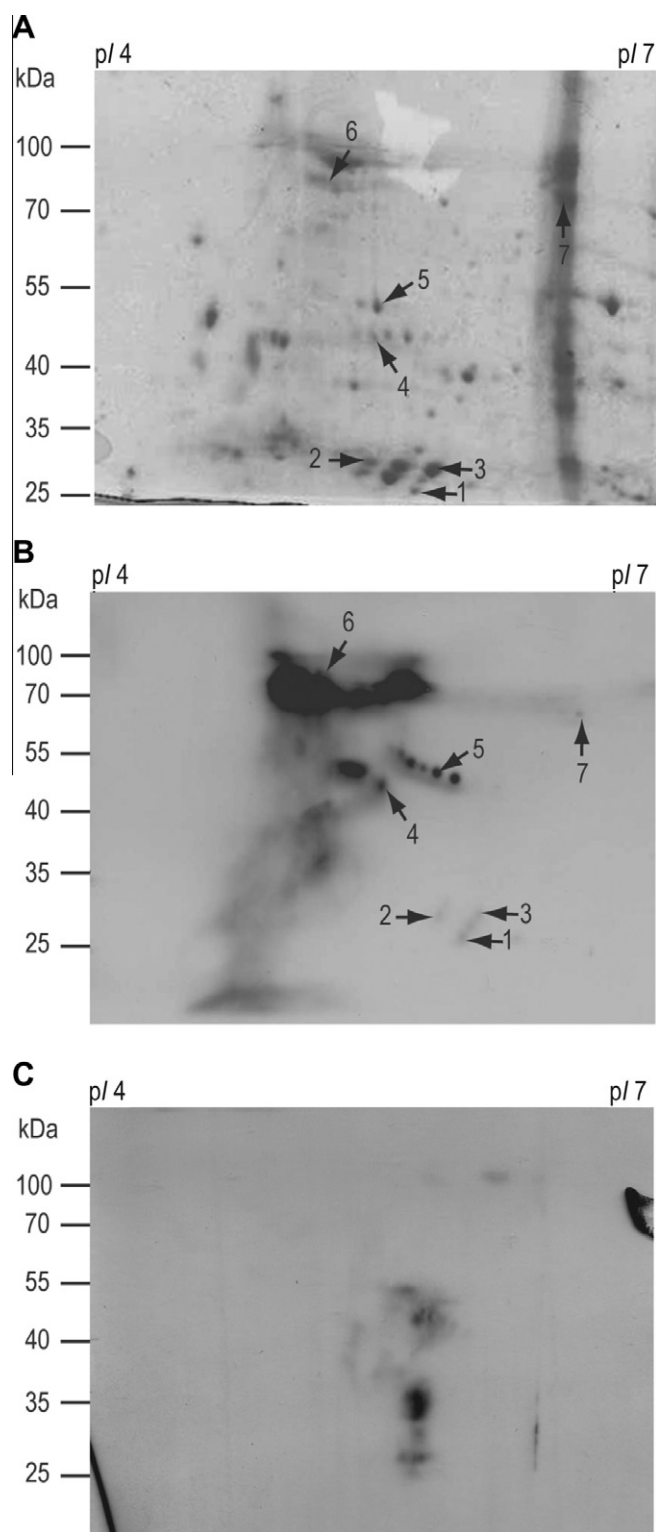
Fig. 1. Identification of immunoreactive proteins from *T. solium* cysticercus by 2-DE Western blot analysis. Total protein was extracted from cysticercus and loaded in the first dimension. Molecular size are indicated on the left (in kDa). Proteins indicated by spot numbers were analyzed by LC–MSMS. (A) A Coomassie Brilliant Blue stained 2-DE gel. (B) A Western blot using sera from patients with NCC. (C) A Western blot using sera from a control group, NCC negative, from the same area.

3.2. Sequencing analysis of Tsol-p27

Analysis of the EST clone BG.R16 revealed a poly (A) tail in the 3′ end at nucleotide 1014. In this gene, an ORF was identified between nucleotides 34 and 744. The protein presents a deduced amino acid sequence of 236 residues with a predicted molecular weight of 26.7 kDa and a theoretical pI of 5.05. A BLAST search using the Tsol-p27 amino acid sequence identified an ORF with similarity to P-29 in *Echinococcus granulosus* (Fig. 2) (Gonzalez et al., 2000).

3.3. SDS–PAGE and Western blot analysis of Tsol-p27 and TsolHSP36

To verify the immunoreactivity, the purified recombinant antigens were separated with SDS PAGE and transferred to nitrocellulose membranes and probed with the NCC-positive and NCC-negative human sera. Western blot strips of purified Tsol-p27 were recognized by antibodies from the positive control group. In contrast, Western blot strips of purified TsolHSP36 were detected only by antibodies present in two serum sample from the NCC-positive group. None of the antibodies present in the sera from the healthy control group reacted to either Tsol-p27 or TsolHSP36 recombinant antigens (Fig. 3).

4. Discussion

Diagnosis of NCC is based on clinical, epidemiological, and laboratory findings, and it can also be accurately performed using neuroimaging techniques such as CT and MRI (Del Brutto, 1997). Unfortunately, these methods are expensive and usually inaccessible in endemic areas, and therefore other protocols are also needed (Diaz et al., 1992). Detection of specific antibodies against *T. solium* cysticerci antigens by ELISA of serum samples from NCC patients has proven to be a useful tool for diagnosing this disease (Deckers and Dorny, 2010). However, the ELISA results can vary significantly, probably because of the heterogeneity of the patients with respect to their immune status and other aspects, as well as differences in the methods used for antigen preparation. In addition, when crude parasite materials are used as a source of antigens, there are a large number of false-positive findings due to cross-reactivity with other parasite antigens (Flisser, 1994). A more reliable technique is the EITB assay, in which antigens are glycoproteins prepared by lectin-affinity purification from an extract of intact cysticerci. Promising EITB results indicating 98% sensitivity and 100% specificity have been reported (Tsang et al., 1989). The disadvantages of EITB are that it uses crude material from the parasite, and it requires special expertise to prepare the antigens and perform the test. Furthermore, this assay is not appropriate for seroepidemiological studies or for use in the ELISA format, cross-reactions can occur in the presence of non-specific fractions (Deckers and Dorny, 2010; Dorny et al., 2003).

The aim of the present study was to identify antigenic proteins and evaluate their diagnostic potential in human NCC. To acquire these soluble proteins from *T. solium*, cysticerci were analyzed by 2-DE Western blotting using NCC-positive human sera from patients in Nicaragua. Six immunoreactive proteins were identified and sequenced by LC–MSMS: phosphoenolpyruvate carboxykinase, 14-3-3 protein in *Echinococcus multilocularis* (Siles-Lucas et al., 1998), actin (Campos et al., 1990), paramyosin (Laclette et al., 1992), small heat shock protein from *T. solium* (TsolHSP36) (Ferrer et al., 2005), and the new protein that we named Tsol-p27. TsolHSP36 and the novel sequence Tsol-p27 were selected for recombinant production and preliminary serological evaluation. The rest of the proteins identified in the current study were not subjected to further analysis due to their conserved nature and

Table 1
Identified immunoreactive proteins recognized by NCC-positive human sera.

Spot No	[a]Protein identity	[b]EST	Amino acid sequence	M_w (kDa)
1,3	Hydatid disease diagnostic antigen P 29 OS. E. granulosus.	TSBG.R16.esd	LGTALEQVASQSEK EFDGLSVQLLDLIR LGTALEQVASQSEK TSDLIHEIDQMK NFLNTTLSEAQK NYYEACAK AKWEAEVR IITATEEFVDINIASK AGELVNKNEK	26.7
2	14-3-3 protein homolog 2. E. multilocularis.	TSAS.R75.esd	AAISTWITDSGC DSTLIMQLLR ICNDVLALLSK YEDMAVAMK	27.7
4	Small heat shock protein p36, T. saginata/T. solium	TSAG.R20.esd	EFHPELEYTQPGELDFLK TQAEIVDGLMVVEAPLFK SVACGDAAMSESVGR GLAIQPSEVQER SIPLPPSVDR VYVHGVTGK EMSAIQPR AEEITIK	35.5
5	OS T. solium GN ACTIN 1PE 3 SV1.	TSAP.R28.esd	LCYVALDFEQEMATAASSSSLEKVAPEEHPVLLTEAPLNPK QEYDESGPGIVHR EITSLAPSTMK GYSFTTTAER AGFADDAPR DLTDYLMK CDVDIR IVAPPER	98.9
6	Phosphoenolpyruvate carboxykinase, putative, Schistosoma mansoni.	TSAU.R48.esd	SEATAAAEFK	71.3
7	Paramyosin OS T. solium GN PMY PE 1 SV2.		TIEELTTTISEMEVR EAAESNLQVSER NAESELNDANGR ELEAELDGEIR IALASEVEEIR GQVGDLTDR YEEEAEAAGNLR YVASQNALNHLK LEGDIGVMQGDLDEAVNARLAAENFELVR MILELQDLLDK LEGLDSQLTR ALTDDLQR	98.8

[a] BLAST search was used with an E-value $<10^{-5}$.
[b] Corresponding EST: name of EST at NCBI database [16].

because they are present in other parasite-related species determined by BLAST search (NCBI). To disregard irrelevant or low BLAST scores a cut off E-value of $1e^{-5}$ was used.

The purified antigens were analyzed by SDS–PAGE, which revealed proteins with molecular weights of 27 and 36 kDa, corresponding to Tsol-p27 and TsolHSP36, respectively. The molecular weight of the recombinant antigens matched the sizes predicted for their native forms and also those measured by LC–MSMS.

Sequencing alignment of Tsol-p27 showed homology with the antigenic protein P-29 previously identified in E. granulosus (Gonzalez et al., 2000). A second start codon at nucleotide 110 was identified in the Tsol-p27 amino acid sequence, which also corresponded to a start codon in E. granulosus P-29 (Fig. 2). If this second codon were used, it would produce a protein with a predicted size of 22 kDa. However, since our LC–MSMS analysis of the native protein demonstrated a molecular weight of 26.7 kDa, it is probable that, in T. solium, the first start codon at nucleotide 34 is used instead of the second start codon.

E. granulosus P-29 is expressed in the larval stage and is considered to be a suitable protein for immunodiagnosis of cystic echinococcosis (CE) in humans (Ben Nouir et al., 2009) . Based on the results of previous studies concerning P-29 immunogenicity, we assume that Tsol-p27 present in the T. solium proteome might play an important role as a diagnostic marker in human NCC.

To be able to compare the immunogenicity of Tsol-p27 with that of TsolHSP36, it was also necessary to clone and express the latter protein. TsolHSP36 has previously been described as a target protein in the diagnosis of human NCC (Ferrer et al., 2005). TsolHSP36 belongs to a family of small heat shock proteins (SHPs) with molecular weights ranging from 12 to 43 kDa and structural features similar to those of the α crystalline proteins, which function as chaperones and are characterized by the presence of conserved homologous sequence of 90–100 residues (Caspers et al., 1995).

To avoid possible recognition of GST by patient antibodies, we removed the GST fusion portion of the recombinant antigens.

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p27  T.solium        MSDLTYQSSIDYPRAGELVINEKTPYATDFGSYPEIDQMK    40
p29  E. granulosus   --------------------------------------MK     2
                                                          **

p27  T.solium        AWLSKIITATEEFVDINIASKAVDACRRNKEKTTSTDKLG    80
p29  E. granulosus   AWLNKIITATEEFVDINMASKVVDAFQKNKEKTTTTDKLG    42
                     ***.************:***.*** :.:******:*****

p27  T.solium        TALEQVASQSEKAAPQLSKMLMEAADVHQRMATAQKEVLN   120
p29  E. granulosus   TALEQVASQSEKAAPQLSKMLMEAADVHQRMATARKS-FN    82
                     **********************************:*. :*

p27  T.solium        SEVNTTFIERFERNFINTTLSEAQKAKAKLEEVRLDLDSD   160
p29  E. granulosus   SEVNTTFIEDLK-NFINTTLSDAQKAKTKLEEVRLDLDSD   122
                     *********  ::  *******:*****:************

p27  T.solium        KSKLKSAKTAEQKAKWEAEVRKDETDFDRVHQESLAIFEK   200
p29  E. granulosus   KTKLKNAKAAEQKAKWEAEVRKDEADFDRVHQESLAIFEK   162
                     *:***.**:*************** :**************

p27  T.solium        TCKEFDGLSVQLLDLIRAEKNYYEACAKECSVMLGE       236
p29  E. granulosus   TCKEFDGLSVQLLDLIRAEKNYYEACAKECS-----       191
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Fig. 2. Alignment of the deduced amino acid sequences of Tsol-p27 and *Echinococcus granulosus* P-29. The symbols indicate identical amino acid residues (∗) and conserved (:) and semi-conserved (.) substitutions.
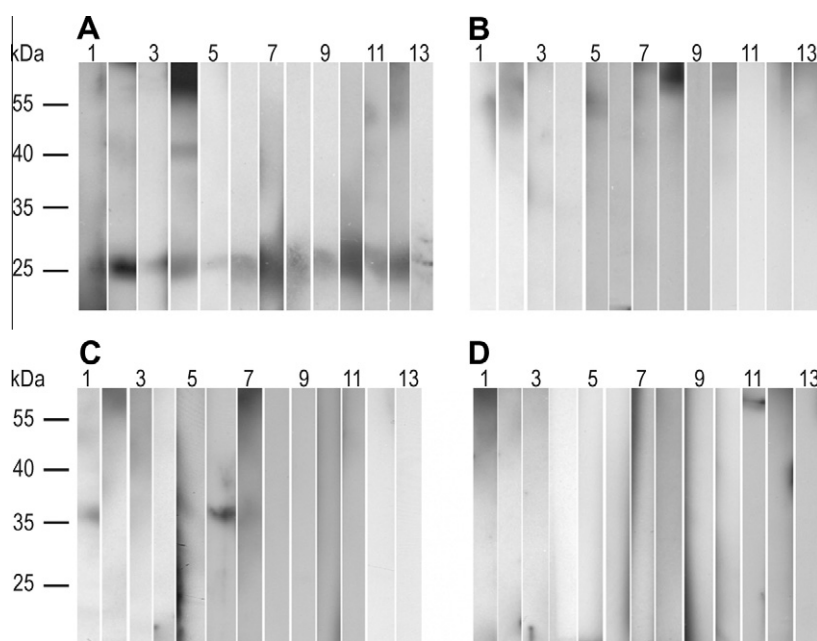
Fig. 3. Immunoblot analysis of recombinant antigens Tsol-p27 (A and B) and TsolHSP36 (C and D). (A–C) Probing done with sera (diluted 1:500) from patients diagnosed as NCC-positive (lanes 1–13). (B–D) Probing done with sera (diluted 1:500) from healthy human subjects (lanes 1–13). Molecular size are indicated on the left (in kDa).

Immunoblotting analysis of Tsol-p27 recombinant antigen was carried out using 13 serum samples from NCC-positive patients and 13 from NCC-negative subjects. TsolHSP36 was recognized by only two of the positive samples, whereas Tsol-p27 was recognized by all 13 positive samples. The immunoreactivity of TsolHSP36 may have been diminished because the recombinant antigen was expressed in *E. coli*, in which glycosylation does not occur. It has been reported that other antigens from *T. solium* lacking glycosylation exhibit reduced antigenicity (Obregón-Henao et al., 2001). Even though the number of serum samples evaluated in our study was limited, the results indicate that Tsol-p27 might be a suitable candidate for diagnosis of human NCC. Complete serological evaluation of both recombinant proteins without GST must be done before any conclusions can be drawn about the utility of Tsol-p27 in diagnosing human NCC. Since none of the proteins were recognized by sera from the NCC-negative group, a combination of both antigens should be evaluated in ELISA settings. Currently, antigens used to diagnose human cysticercosis still rely on a parasite extract or multi-component glycoprotein fractions, despite the difficulties related to obtaining parasite material and the moderate sensitivity and specificity of the tests (Dorny et al., 2003).

Improvement of immunodiagnostic tests will facilitate a better understanding of the prevalence and epidemiology of NCC in ende-

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mic areas (Deckers and Dorny, 2010). Here, we present a new immunogenic protein, Tsol-p27, which shows promise as a diagnostic antigen, used alone or in combination with TsolHSP36.

Acknowledgments

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