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Immunodiagnosis of porcine cysticercosis: Identification of candidate antigens through immunoproteomics

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ABSTRACT

Cysticercosis, caused by the larval stage of *Taenia solium*, is a zoonotic disease affecting pigs and humans that is endemic to developing countries in Latin America, Africa and South East Asia. The prevalence of infection in pigs, the intermediate host for *T. solium*, has been used as an indicator for monitoring disease transmission in endemic areas. However, accurate and specific diagnostic tools for porcine cysticercosis remain to be established. Using proteomic approaches and the *T. solium* genome sequence, seven antigens were identified as specific for porcine cysticercosis, namely, tropomyosin 2, alpha-1 tubulin, beta-tubulin 2, annexin B1, small heat-shock protein, 14-3-3 protein, and cAMP-dependent protein kinase. None of these proteins were cross-reactive when tested with sera from pigs infected with *Ascaris* spp., *Cysticercus tenuicollis* and hydatid cysts of *Echinococcus* spp. or with serum from a *Taenia saginata*-infected cow. Comparison with orthologues, indicated that the amino acid sequences of annexin B1 and cAMP-dependent protein kinase possessed highly specific regions, which might make them suitable candidates for development of a specific diagnostic assay for porcine cysticercosis.

Introduction

Cysticercosis is a parasitic disease caused by the larval stage of the tapeworm *Taenia solium* (cysticerci) that affects pigs and humans and which prevails as a major human health and veterinary problem in developing countries of Latin America, Africa and Asia. In Mexico, taeniasis/cysticercosis is associated with low socio-economic conditions; a recent study, performed in the major neurological hospital of Mexico, indicated that the prevalence of human neurocysticercosis was relatively stable between 1994 and 2004 (Sciutto et al., 2000; Fleury et al., 2011).

Tongue inspection is still the major procedure for detection of porcine cysticercosis in the field. However, it requires participation of trained personnel and demonstrates poor sensitivity (Sciutto et al., 1998a). A serological assay would be a convenient alternative for diagnosis of porcine cysticercosis. Previously, a number of immunodiagnostic methods have been designed, based on different extracts of parasite antigens, using ELISA or electro-immunoblot transfer (EITB) techniques (Tsang et al., 1989; Dorny et al., 2003; Prabhakaran et al., 2007; Atluri et al., 2009, 2011). In spite of some encouraging results obtained using EITB, this is a costly and labour-intensive procedure, which limits its use in epidemiological surveys. Several parasite antigens, such as the vesicular fluid, the secretion/excretion products or crude extracts from *T. solium* cysticerci and related parasites, like *Taenia crassiceps* and *T. saginata*, have been used for detection of host antibodies. Results have shown variable sensitivities and specificities, depending mainly on the severity of the infection in individual pigs (Arruda et al., 2005).

New proteomic tools are now available that allow high resolution analysis of complex groups of proteins, through two-dimensional gels (2D-PAGE). The resolved protein can be blotted and tested for immunoreactivity with different sera. Finally, isolated proteins can be individually identified through mass spectrometry. The aim of the current study was to perform a 2D-PAGE/EITB to identify novel antigens that would allow development of a more sensitive and specific assay for porcine cysticercosis.

Materials and methods

Parasites and protein extracts

Taenia solium cysticerci were obtained from skeletal muscle of naturally-infected pigs and extensively washed with sterile 0.15 M phosphate-buffered saline (pH 7.2; PBS). Parasites were resuspended in approximately three volumes of lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 10 mM Tris and protease

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inhibitors (0.5 M EDTA, 200 mM PMSF, 10 mM leupeptin and 1 mM pepstatin). The material was then homogenized on ice and centrifuged at 14,000 g for 15 min. The soluble antigens in the supernatant were recovered, quantified with 2-D Quant kit (Amersham), and frozen at −70 °C until use.

Animals and sera

Pools of serum samples from naturally-infected *T. solium* cysticercotic pigs, or from pigs infected with other parasites (*Ascaris* spp., *Cysticercus tenuicollis*, hydatid cysts of *Echinococcus* spp.), as well as a single serum sample from a calf that was experimentally infected with gravid proglottids of *T. saginata*, were obtained. Sera from non-cysticercotic pigs that reacted positive to the crude antigenic extract were also obtained, to be used in the analysis as false positives (Sciutto et al., 1998b). Finally, sera from pigs that had been confirmed as uninfected for all the parasites described above were also obtained, to be used as negative controls. In all cases, with the exception of the calf serum, pools were made using at least five different individual sera. All animals employed in this study were necropsied to certify infection status. This research study was approved by the Ethical Committee of the Veterinary Medicine School (UNAM).

Two dimensional polyacrylamide gel electrophoresis

Isoelectric focusing (IEF) was performed in 17 cm IPG strips (Bio-Rad) with non-linear pH gradients 3–10 or 3–6 using a Protean IEF Cell (Bio-Rad), with constant surface temperature of 20 °C and maximum current of 50 μA/strip. Samples of 150 μg of crude antigenic extract, were diluted in 125 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% IPG buffer of the respective pH gradient and bromophenol blue). After applying the samples to the IPG strips, these were rehydrated at 20 °C for at least 12 h. IEF was run for a total of 10,000 V h. Afterwards, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris pH 8.8, 2% SDS and 30% glycerol) containing 2% DTT for 15 min and then alkylated in equilibration buffer containing 2.5% iodoacetamide for 15 min.

The second dimension was performed on 12% SDS–polyacrylamide gels using a Mini Protean cell (Bio-Rad). After running at 200 V, the 2-D gels were either stained or electroblotted onto nitrocellulose membranes for immunoblot analysis. Reproducibility of the proteomic pattern was verified by running the 2-D gels in triplicate at different times. The 2-D gels were routinely stained with silver stain, or with Coomassie blue when the gel was used for mass spectrometry (MS) analysis. Analysis of 2-D gels was achieved using PDQuest 2-D Analysis software (Bio-Rad).

Immunoblot analysis

Proteins were electro-transferred from 2-D gels to nitrocellulose membranes at 100 V for 90 min. Membranes were initially blocked by overnight incubation at 4 °C with 10% dried skimmed milk in PBS buffer and then incubated for 2 h at 37 °C with one of the pooled sera pig samples or the single calf sera described above (diluted 1:20 in the same buffer). The membrane was washed three times with 0.1% Tween 20 in PBS (PBST) and incubated again for 2 h at 37 °C with a horse-radish peroxidase conjugated anti-porcine IgG antibody (Sigma) diluted at 1:500 in PBST. After washing as above, immunoblots were developed by standard procedures with 4-chloro-1-naphthol used as the substrate. Reproducibility of immune recognition was verified by repeating the immunoblot at least three times.

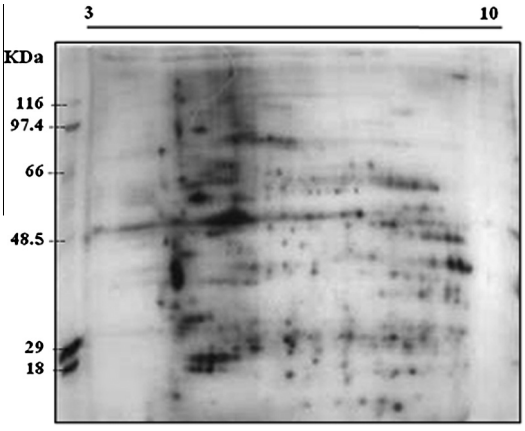

Fig. 1. Representative 2-DE gel of *T. solium* cysticerci proteins. Proteins (150 μg) were separated with IPG strip of 17 cm, on a non-linear pH range of 3–10. The proteins were visualized with silver staining.

Mass spectrometry (LC/MS/MS)

For protein identification, spots of antigenic proteins were carefully excised from Coomassie blue-stained 2-DE gels and prepared for LC/MS/MS. Individual protein spots were destained, reduced, carbamido-methylated, digested with trypsin, and extracted from the gel using a standard in-gel digestion procedure (Kinter and Sherman, 2000). The volume of the extracts was reduced by vacuum centrifugation at room temperature, then adjusted to 20 μL with 1% formic acid.

Peptide mass spectrometric analysis was carried out on a 3200 QTRAP System (Applied Biosystems/MDS Sciex), with a nanoelectrospray source and a nanoflow LC system (Agilent 1100 Nano Pump, Waldbronn). Mass tuning of the hybrid triple quadrupole linear IT spectrometer was performed with [Glu1]-fibrinopeptide B. Antigen sample digests were injected on a Zorbax 300SB C18 column (3.5 mm, 5060.075 mm^2, Agilent) equilibrated with 2% acetonitrile, 0.1% formic acid and separated using a linear gradient from 2% to 70% acetonitrile, 0.1% formic acid over an 80 min period, at a flow rate of 300 nL/min. The interface heater for desolvation was held at 150 °C. Spray voltage was 2.4 kV.

Spectra were acquired in automated mode using information dependent acquisition (IDA). Precursor ions were selected in Q1 using the enhanced MS mode. The scan range for EMS was set at m/z 400–1500 and 4000 amu/s. Selected ions were subjected to an enhanced resolution scan at the low speed of 250 amu/s over a narrow (30 amu) mass range and then to an enhanced product ion scan (MS/MS). The precursor ions were fragmented by collisionally activated dissociation (CAD) in the Q2 collision cell using rolling collision energy. The fragment ions generated were captured and mass analyzed in the Q3 linear IT. Database searching (Swiss-Prot, NCBInr or MSDB) and protein identification were performed with MASCOT software,[2] with trypsin plus one missed cleavage, carboxyamidemethylation as a fixed modification and methionine oxidation as a variable modification and a mass tolerance of 0.5 Da for the precursor molecular weights and 0.3 Da on the fragment molecular weights.

The criteria to accept a protein hit as a valid identification were: two or more tryptic peptide matches to the protein sequence and at least one peptide with $P < 0.05$. Partial sequences obtained from the MS analysis were used for screening of full-length sequences in the *T. solium* genome database (Tsai et al., 2013) using the basic local alignment search tool (BLAST[3]).

Results

2-D PAGE of T. solium cysticerci whole extracts

Three hundred and fifty protein spots were identified in the silver stained 2-DE gels, with molecular masses ranging from 12.6 to 180 kDa and pI ranging from 4 to 10 (Fig. 1). The majority of spots were found between 18–86 kDa and pI: 5.0–8.7.

Identification of antigenic proteins

Proteins of potential interest for diagnosis of cysticercosis were identified by immunoblotting. For this purpose, a group of pig sera from naturally infected pigs (*T. solium*, *Ascaris* spp., *C. tenuicollis* or *Echinococcus* spp.) as well as from a calf (experimentally infected with gravid proglottids of *T. saginata*), were assessed against the *T. solium* crude extract as described previously. We also used sera from non-cysticercotic pigs that reacted positively or negatively to the *T. solium* crude extract in previous tests.

Forty-three spots were recognized by immunoblot when the pool of sera form cysticercotic pigs (20.5–150 kDa and pI: 3.6–8.0) was assessed, using gels with pH ranges of 3–10 and 3–6, against the *T. solium* crude extract (Fig. 2). After extensive testing for cross reactivity, using all other sera from infected and healthy animals, only 13 spots (Fig. 2) were shown to be recognized exclusively by the sera from cysticercotic pigs (21.6–47.2 kDa and pI: 4.7–5.4). A group of strongly-reactive spots, aligned at 50 kDa with pI varying between 3.6 and 7.5, gave consistently positive results in all tests (Fig. 2). These spots were found to correspond to pig IgG immunoglobulin, which are normally taken up by cysticerci from the host (Ambrosio et al., 1994) and are thus recognized by the secondary antibody (data not shown).

Thirteen of the antigenic spots exclusively recognized by sera

[2] See: http://www.matrixscience.com.
[3] See: http://blast.ncbi.nlm.nih.gov/Blast.cgi.