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Characterization of a novel *Taenia solium* oncosphere antigen

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Abstract

Infections due to *Taenia solium* in humans (taeniasis/cysticercosis) remain a complex health problem, particularly in developing countries. We identified two oncosphere proteins that might protect the porcine intermediate host against cysticercosis and therefore help prevent disease in humans. One of these proteins was further identified by two-dimensional gel-electrophoresis and micro-sequencing. The gene encoding this protective protein was also identified, cloned and characterized. The native 31.5 KD protein *Tso31* has four variants at the cDNA level. The longest sequence from which the others seem to derive, encodes a 253 amino acid peptide. The predicted protein has a molecular weight of 25.1 KD, one putative N-glycosylation site, two fibronectin type III domains, and one C terminal transmembrane domain. The gene structure of the protein consists of four exons and three introns. The finding of one gene and four different cDNAs for *Tso31* suggests the existence of a possible mechanism of differential splicing in this parasite. The *Tso31* protein is exclusive to *T. solium* oncospheres with a putative protein structure of an extra-cellular receptor-like protein. The *Tso31* protein was expressed as a recombinant protein fused to GST and tested in a vaccine to determine its effectiveness in protecting pigs against cysticercosis. Only two pigs out of eight vaccinated were protected and although the total median number of cyst decreased in vaccinated pigs compared to controls this decrease was not statistically significant ($P=0.09$).

Keywords

Taenia solium; cysticercosis; taeniasis; cDNA; protein sequencing

INTRODUCTION

The parasite, *Taenia solium*, infects humans and swine causing disease and economic loss. Humans are the only known definitive host and pigs are the obligate intermediate host for the life cycle of the parasite, harboring the larval stage. Infection of pigs with the larval stage is termed cysticercosis and follows ingestion of proglottids and eggs present in faeces from people harboring the adult tapeworm. Taeniasis is the infection of people with the adult tapeworm that

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follows ingestion of larval cysts in infected pig meat. Taeniasis is a disease exclusive to humans, however, cysticercosis is not exclusive to pigs; it also affects humans who may act as an accidental intermediate host. In humans, the larval stage often infects the central nervous system (CNS) producing a clinical disorder known as neurocysticercosis (NCC). [1,2].

In developing countries where pig husbandry is common, *T. solium* is probably responsible for over 10% of acute care admissions to neurological wards [3–5] and nearly 30% of late onset seizures are associated with serological evidence of infection [4]. In endemic regions 25 to 39% of pigs may be infected [6,7].

No control strategy has been demonstrated to be effective and sustainable except the improvement in public health that accompanies development [8,9]. The essential role of pigs as obligatory intermediate hosts presents a possibility of interfering with transmission of both diseases (taeniasis and cysticercosis) through vaccination of pigs. To date, oncosphere antigens are the most effective vaccine candidates in preventing cysticercosis [10–15]. We identified two oncosphere protein bands in western blot that were associated with immune protection against cysticercosis in pigs [12]. The cDNA for one of these proteins, Tso31, was cloned and the corresponding gene characterized. The Tso31 protein was also expressed as a recombinant, fused to the GST protein and tested in a vaccine trial to prevent cysticercosis in pigs.

MATERIALS AND METHODS

Tapeworm recovery and oncosphere preparation

Tapeworm fragments were recovered from post-treatment stools as described previously [16, 17]. Tapeworms were identified at the species level by polymerase chain reaction and restriction enzyme analysis (PCR-REA) [18]. *T. solium* eggs were hatched *in vitro* using sodium hypochlorite (0.75%) for 10 min as described [12,19]. Oncospheres were washed three times in RPMI 1640 media (Sigma, St. Louis, MO) by centrifuging at 3000× g for 5 min at 4°C and counted using a Neubauer chamber. After the final wash RPMI was completely removed and oncospheres were re-suspended either in 8M urea for two-dimensional gel electrophoresis or in Trizol (Invitrogen, Carlsbad, CA) for RNA purification.

Two-dimensional electrophoresis

After re-suspending the oncospheres in 8M urea, a protease inhibitor cocktail was added at the following final concentrations: pepstatin (1.0 µg/ml) (Calbiochem, La Jolla, CA), leupeptin (1.0 µg/ml) (Calbiochem), and Pefabloc (1mM) (Roche Applied Science, Indianapolis, IN). Re-suspended oncospheres were then sonicated in a bath type sonicator for 15 min at 37°C.

The first dimension isoelectrofocusing (IEF) gel was performed using IPGphor (Amersham Biosciences, Piscataway, NJ). A total of 35 µg of protein was prepared in a re-hydration solution containing 8 M urea, 2% Triton X-100, 0.05% IPG buffer pH 4–7, and 0.002% bromophenol blue. IPG strips were re-hydrated for 12 hours, run at 130 V for 2 hrs, 500 V for 1 hr, 1000 V for 1 hr, 8000 V for 2 hrs. The strips were then equilibrated in SDS equilibration buffer containing 50 mM Tris-H₂SO₄ pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% SDS (w/v) and 0.002% bromophenol blue for 15 min and loaded onto 1 mm thick 5–22.5% SDS-PAGE gradient gels for the second dimension. Second dimension SDS-PAGE and western blot were performed as described [20,21].

Protein sequencing

After two-dimensional gel electrophoresis, acrylamide gels were directly stained with colloidal Coomassie blue (Invitrogen). Protein spots were identified by comparing the Coomassie stained gel with a second gel which was processed at the same time and under exactly the same

conditions but was transferred to nitrocellulose and probed with sera from protected pigs vaccinated with crude oncosphere extracts. For sequencing, the specific protein spots were excised and pooled from three different gels. Protein samples excised from gels were reduced, carboxyamidomethylated, digested with trypsin and analyzed by μ LC/MS/MS. The resulting MS/MS spectra were correlated with known sequences from protein and nucleotide databases using the Sequest algorithm. Any uncorrelated MS/MS spectra from two-dimensional gel samples were manually *de novo* sequenced to obtain novel peptide sequences [22]. Protein sequencing was done at the Harvard Microchemistry and Proteomics Analysis Facility.

***T. solium* oncosphere cDNA library construction**

Freshly hatched oncospheres were pooled to a final count of 3×10^6 and re-suspended in 1 ml of Trizol (Invitrogen) for total RNA extraction. Poly (A) RNA was recovered using Poly (A) Quik mRNA Isolation Kit (Stratagene, La Jolla, CA). The cDNA synthesis was performed using 0.55 μ g of poly A⁺ RNA and the ZAP-cDNA synthesis kit (Stratagene). The cDNA was cloned into the lambda Uni-ZAP XR vector followed by packaging of the lambda DNA using the Gigapack® III Gold Cloning Kit (Stratagene) according to manufacturer's instructions.

Partial cDNA cloning and library screening

Degenerate primers were designed from sequenced peptides using a *T. solium* codon bias table [23]. DNA primers were synthesized by the Centers for Disease Control (CDC) DNA core facility (Atlanta, GA). AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA) was used in all PCR reactions. PCR products were generated using an annealing temperature of 45 °C for 40 cycles. The cDNA library described above was phenol-chloroform extracted and used as the DNA template. Two degenerate oligonucleotides, (OA2F3a: 5'-CAY TGG GGI CCI MTI ACI GCI AA-3' and OA2R1a: 5'-AKR TTY TCI GTR AAR TCR AAR TA-3'), corresponding to the amino acid sequence YFDFTENL and HWGPLTAK respectively, amplified a product of 250 bp. The amplification product was cloned into the pCR 2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen) for DNA sequencing. Five different clones were sequenced.

The PCR product of the partial cDNA clone was radioactively labeled using α -[³²P]-dCTP by random priming according to the manufacturer's protocol (Invitrogen). Radioactive labeled probes were then used to screen the oncosphere cDNA library. Four clones from positive plaques were sequenced.

RACE PCR and generation of full-length cDNA

To identify the 5' end of the Tso31 cDNA sequence, 5' end RACE- PCR was performed with specific primers using 0.5 μ g of total RNA and the SMART RACE cDNA amplification kit from Clontech (Palo Alto, CA) according to the manufacturer's protocol. To corroborate the full-length cDNA sequence of the Tso31, PCR was carried out using primers designed to complement the 5' and 3' ends of the assembled cDNA sequence. Primer pairs were, Tso31-F: 5'-ACC GGG ATG CAT GCG CAG TGT GAA GAT -3' and Tso31-R: 5'-TCG TGT ATT TGT CGT GCG GGT CTA C -3'. The 5'-ready-cDNA prepared for the RACE-PCR was used as the template. Amplification products were then cloned into the pCR 2.1 TOPO vector (Invitrogen), four clones were sequenced.

Tso31 DNA gene structure analysis

To obtain gene sequence data, *T. solium* genomic DNA was extracted as previously described [18]. Primers Tso31-F and Tso31-R were used to amplify the genomic DNA. PCR amplification was carried out with AmpliTaq Gold DNA polymerase (Applied Biosystems)

using an annealing temperature of 55°C for 40 cycles. Amplicons were cloned into the pCR 2.1 TOPO vector using the TOPO TA cloning kit (Invitrogen) for DNA sequencing.

Identification of Tso31 protein using antibody produced against rTso31

The Tso31a cDNA sequence encoding the mature protein, without the signal peptide sequence, was cloned into the expression vector pGEX-4T-1 (GE Healthcare, Piscataway, NJ). Protein was expressed in BL21 Star™ (DE3) *E. coli* (Invitrogen) according to manufacturer's protocol. Expressed protein was purified using a GSTrap™ HP Column (GE Healthcare). The purified protein was desalted into PBS pH=7.48 and used to immunize rabbits.

To determine if the Tso31 protein is exclusive to *T. solium* oncospheres western blot was performed using 0.05 ng/mm of rGST-Tso31a and 0.1ng/mm each of oncosphere, cyst, and tapeworm crude antigens. Rabbit immune sera were diluted 1:100 in 5% milk-PBS. The blot was processed then as previously described [20;21]. To corroborate that the protein recognized in the oncosphere extract was effectively the Tso31 protein and not a cross reaction due to the fusion to the GST protein, a second blot was performed using a 1:500 dilution of the immune sera and blocked for one hour using 1.5 ug of rGST before adding the strip containing the antigen.

Indirect immunofluorescence

Oncospheres were activated by incubation with artificial intestine fluid for one hour at 37°C, washed twice with RPMI media, incubated with 10% fetal bovine serum-RPMI at 37°C for 10 min and washed three times with PBS pH=7.4 (five minutes each). Activated oncospheres (about 400) were fixed on poly-L-lysine coated multiwell slides by drying at 37°C. Fixed oncospheres were alternatively permeabilized with 0.01% Triton X-100 in PBS for 30 seconds. Oncospheres were then incubated in 1% albumin from chicken egg white (Sigma) PBS for 30 min, washed and incubated with primary antibody (rabbit anti-rGST/Tso31 antibody) at 1:2000 in 1% albumin-PBS for 60 min. After washing the oncospheres were incubated in a 1:100 dilution of goat anti-rabbit IgG (H+L) fluorescein labeled conjugate (Kierkegaard & Perry Laboratories, Gaithersburg, MA) in PBS + 1% ovalbumine, 0.35% Evans Blue, for 60 min. After the final wash oncospheres were observed using a fluorescence microscope (Nikon, Melville, NY) and photographed using a Sight DS-5M-L1 digital camera (Nikon). Cross sections of cysts from naturally infected pigs and proglottids from tapeworms obtained from hamsters were similarly subjected to immunofluorescence using rabbit anti-rGST-Tso31 sera at 1:500 to observe the presence of the Tso31 protein.

Pig vaccine trial

Twelve two month old pigs (Pietrain/Landrace mixed breed) were used in the vaccine trial. The pigs were vaccinated with two doses (two weeks apart, 0.5 mg each) of either rGST or rGST-Tso31 that were given with 1 mg of Quil A as adjuvant (Superflos Biosector, Vedbaek, Denmark). Eight pigs received rGST-Tso31, four pigs received rGST. Fifteen days after the final vaccination all pigs were challenged with one gravid proglottid obtained from tapeworm carriers as described [16,17]. Twelve weeks after challenge, pigs were humanely sacrificed and the entire carcass and brain were assessed for presence and viability of cysticerci [12,15]. Cyst were classified as viable if a defined cyst structure with liquid content was still present, and degenerated if this has been replaced by a semisolid contents or inflammatory scar [15]. Antibody response was assayed by ELISA using 2 µg/ml of recombinant GST-Tso31 protein [24].

DNA sequencing and analysis

cDNA sequencing was performed at the Emory University DNA core facility (Atlanta, GA). Genomic DNA sequencing was performed at the Molecular Biology laboratory at the Instituto de Medicina Tropical Alexander von Humboldt, Universidad Peruana Cayetano Heredia (Lima, Peru). Data from DNA sequencing were assembled and analyzed using SeqMan software (DNASTAR, Madison, WI) and other software including Generunner (www.generunner.com), BLAST (www.ncbi.nlm.nih.gov/BLAST/), Signal P (www.cbs.dtu.dk/services/SignalP), SMART (<http://smart.embl-heidelberg.de/>), Splign (<http://www.ncbi.nlm.nih.gov/sutils/splign/>), and Prosite (www.expasy.org/prosite).

Nucleotide sequence accession numbers

The nucleotide and deduced amino acid sequences of Tso31 have been entered into GenBank. Tso31a accession number is **AY752889**; Tso31b accession number is **AY752890**; Tso31c accession number is **AY752891**; and Tso31d accession number is **AY752892**. Tso31 gene sequence accession number is **DQ861410**.

RESULTS

Protein sequencing

Three protein spots (OA1–OA3) corresponding to the 31KD protein were identified and sequenced (Fig 1). Using *de novo* MS/MS sequencing, a total of five novel peptides were identified from spot OA2. The *de novo* MS/MS sequencing was not fully performed for spot OA3 but the two peptides obtained (TTILELKK and TTTSEPFSGKGT) were identical to those observed in OA2. In addition, the protein spectra of spot OA3 was almost the same as the spectra for spot OA2. These data suggest that the OA2 and OA3 are derived from the same 31 KD protein backbone but with different pIs. No novel sequences were observed in protein spot OA1. Possible protein contamination was observed in protein spot OA1; spectra of at least five other proteins were also present in this spot (data not shown).

Characterization of Tso31 cDNA

PCR amplification of the cDNA library using primer pair OA2-F3a and OA2R1a gave a 250 bp product. Sequence analysis showed that this product encodes three of the five peptides observed by *de novo* sequencing. Clones of 1600 bp were identified from library screening using this amplification product as a probe. All sequenced clones were identical. The clones contained a 900 bp incomplete open reading frame (ORF) lacking an initiating ATG. The 5' RACE PCR identified two likely ATG start codons and showed the presence of a signal peptide sequence on the 5' end of the cDNA sequence (Fig S1 & S2).

The inferred full length ORF was confirmed by PCR amplification of the Tso31 cDNA using primers that complemented the 5' end and the 3' end of the assembled cDNA. This amplification resulted in the identification of four complete clones (Tso31 a–d). The longest Tso31 clone (Tso31a) comprises 990 nucleotides with an open reading frame of 762 nucleotides. The three other clones seem to be derived from Tso31a, essentially the cDNA sequence was the same as that of Tso31a, except that fragments of 288 (Tso31b), 192 (Tso31c), and 291 (Tso31d) nucleotides were missing at different positions. Clones Tso31b, Tso31c, and Tso31d comprise 703, 799, and 700 nucleotides with ORFs of 474, 570, and 471 bp respectively (Fig S1 & S2).

Sequence analysis and homology searches

Amino acid sequence analysis of the deduced protein showed that the ORFs derived from Tso31a, Tso31b, Tso31c and Tso31d encode peptides of 253, 157, 189, and 156 amino acids respectively. All four clones possess the 21 amino acid signal sequence located at the N-

terminus as predicted by the Signal P software. The primer Tso31-F included the first five amino acids of this signal sequence. The longest mature Tso31 protein (Tso31a) has a predicted molecular weight of 25.1 KD and a predicted isoelectric point of 5.08. Predicted isoelectric points of Tso31b, Tso31c and Tso31d were 5.23, 4.78, and 5.66 respectively. PROSITE analysis showed that Tso31a possesses one potential N-glycosylation site. The N-glycosylation site is absent in the variant clone Tso31b but present in Tso31c and Tso31d (Fig S1).

SMART analysis showed that Tso31a has two fibronectin type III domains (amino acids 30–110 and 127–208) and a transmembrane domain (amino acids 233–252). In spite of lacking part of the peptide sequence, each of the other variant proteins (Tso31b, Tso31c, and Tso31d) retains one fibronectin type III domain with the signal peptide and transmembrane domain unchanged (Fig S2).

Tso31 gene structure

Sequencing data from amplified genomic DNA were assembled and aligned using SeqMan software (DNASTAR). No sequence differences were observed when all clone sequences were aligned.

Tso31 gene sequence was aligned to each of the Tso31 cDNA sequences (Tso31a, Tso31b, Tso31c and Tso31d) using the software Splign to identify exon/intron boundaries (Fig S3). The DNA sequence of the gene compared to full length Tso31 cDNA (Tso31a) shows the presence of three introns, all with the predicted splice sites containing the 5' GT and 3' AG rule at the splice junctions [25]. The putative exon/intron structure of the Tso31 gene consists of four exons and three introns. The predicted exons for Tso31a were 95, 288, 291, and 300 nucleotides respectively.

With the exception of the Tso31c variant, both the 5' end and 3' end of the sequences absent in the variant cDNAs match perfectly with the 5' and 3' ends of the intron sequences. Splicing of Tso31c differs from that observed in the other variants. The 5' and 3' ends of the second intron do not match with introns of the other variants. Prediction with Splign shows a GC pair at the 5' end and an AA pair at the 3' end of the second intron.

Antibody identification of cloned protein

The identity of the characterized protein was corroborated using antibodies raised in rabbits against recombinant GST-Tso31. As observed in figure 5, antibodies raised against rGST-Tso31 recognize a single protein band in the crude oncosphere antigen. The protein recognized by antibodies against rGST-Tso31 comigrates with the protein recognized by sera from pigs vaccinated with crude oncosphere antigens protected against cysticercosis. When the immune rabbit serum was blocked with rGST, the intensity of the reaction remained unchanged, indicating that the antibodies were specifically recognizing the Tso31 protein in the oncosphere (data not shown). No protein bands were recognized by the rabbit anti-rGST-Tso31 serum when either adult tapeworm or cyst crude extracts were used as antigen (Fig 2).

Indirect Immunofluorescence

The indirect immunofluorescence (IFI) of oncospheres using antibodies raised against rGST-Tso31 in rabbits recognized the native Tso31 protein on the oncosphere (Fig 3). The Tso31 protein seems to be localized on the limiting membrane of activated oncospheres. When oncospheres were treated with Triton X-100 the fluorescence remained confined to the limiting membrane of the oncosphere with no fluorescence of the inner structures of the oncosphere. Membrane fluorescence for the Tso31 protein increase as activation occurred. Sections of cyst and proglottids of tapeworms obtained from hamsters did not show the presence of the Tso31 protein by indirect immunofluorescence (data not shown).

Pig vaccine trial

Out of the eight pigs vaccinated with the rGST-Tso31 protein only two pigs were completely protected. However, one of them had degenerated cysticerci. Although there was a tendency for reduced numbers of cysticerci in the vaccinated group (median= 860.5 cysts/pig) compared to the controls pigs vaccinated with rGST (median= 2348 cysts/pig), this difference did not reach a statistical significance ($P=0.0894$, Mann Whitney test) (Table 1). No differences were observed in the antibody response to the recombinant GST-Tso31 protein among the eight vaccinated pigs. After the second vaccine dose the antibody level decreased with time. The *T. solium* infection in these pigs did not have a booster effect on the antibody response (Fig 4).

DISCUSSION

A novel *T. solium* oncosphere protein has been characterized and described in this study. Two-dimensional gel electrophoresis was employed for protein purification followed by *de novo* MS/MS for peptide sequencing because the amount of oncosphere protein available was very small.

Although three protein spots were identified by western blot of a two dimensional gel, peptide sequences corresponding to the Tso31 protein were identified only from two different spots (OA2 and OA3) by *de novo* MS/MS analysis. In spot OA1, the presence of higher concentrations of other proteins probably masked the presence of Tso31 or its mass spectra. It is also possible that Tso31 was in fact absent in this spot and that the other proteins contributed to the immuno-activity of this protein spot.

The Tso31a cDNA sequence showed that all peptides derived from sequencing belonged to the Tso31 protein. At the cDNA level four variants of the protein were observed, three of the variants (Tso31b, Tso31c, and Tso31d) seem to derive from Tso31a, which is the longest variant. However, it is not clear if Tso31b, Tso31c and Tso31d are translated into proteins. The potential N-glycosylation site present in the protein may explain the difference in molecular size between the native protein (31KD) and the sequence predicted from the cDNA sequence (25.1KD). The differences in the pIs, as noted between OA2 and OA3, may be ascribed to glycosylation or other posttranslational modifications.

Two different *T. solium* recombinant oncosphere proteins (Tsol18 and Tso145) have been tested and shown to be highly effective in protecting pigs against cysticercosis [14,15]. Both proteins plus a third, Tso116, have been identified and cloned by homology to the *T. ovis* and *T. saginata* protective antigens [26;27;]. All of these oncosphere proteins present highly conserved regions with a putative secretory signal and with one or two fibronectin type III domains in each [28].

Tso31 shares about 40% amino acid identity with the Tso145 protein family. The Tso31 protein structure resembles the structure of the Tso145 family and the host protective 45W antigen of *T. ovis* consisting of one transmembrane domain with one or two fibronectin domains [27]. This is contrary to that observed for *T. solium* antigenic proteins Tso118 and Tso116, which are secreted proteins with a single fibronectin domain [13,29]. These observations suggest that Tso31 protein might be anchored in the cell membrane with one or two exposed fibronectin domains.

Fibronectin molecule appears to be important in mediating adherence of bacterial pathogens and parasites such as *Trichomonas vaginalis* and *Trypanosoma cruzi* [30–33]. Likewise fibronectin III domains might be important for cell adhesion in cestodes [13,34].

No differences were observed at the DNA level in any of the four clones sequenced for each sample suggesting that the gene is conserved similar to that observed for the Tso18 gene [35]. The gene structure of Tso31, which consists of four exons and three introns, also resembles that observed for Tso145 antigens in *T. solium* [26], the Tsa9 antigen in *T. saginata* [13] and the To45W antigen in *T. ovis* [36]. The presence of cDNA variants for this protein strongly suggests the existence of alternative splicing for this protein as observed previously in the *T. solium* Tso145 antigen family [26].

We have shown that Tso31 is exclusively expressed in the oncosphere stage; since the protein was not detected by Western blot or by indirect immunofluorescence in the cyst or the tapeworm. The Tso31 protein seems to be present predominantly on the membrane of the oncosphere. The intensity of fluorescence was reduced due to the effect of Triton X-100 on the oncospherical limiting membrane but the internal structures of the oncosphere showed no fluorescence. The presence of the Tso31 protein seems to increase with the activation of the oncosphere since non-activated oncospheres did not fluoresce at all, while fully activated oncospheres fluoresced most intensely both in non-permeabilized and permeabilized oncospheres (Fig 3).

Native Tso31 is exclusively recognized by sera from pigs protected against cysticercosis vaccinated with crude oncosphere antigens. However the vaccine trial using rGST-Tso31 failed to confer significant protection against cysticercosis in pigs in contrast to the closely related protein Tso145, which has been shown to be a successful vaccine [15]. Several reasons might contribute to the failure of protection by this protein. The prokaryotic expression system probably does not fold the protein properly. The presence of the transmembrane domain in the recombinant protein might also contribute to the improper folding. In addition, protein glycosylation might be important for Tso31 to confer protection.

Mass chemotherapy of both people and pigs to interrupt the transmission of *T. solium* in developing countries has been tried and is of limited efficacy. The addition of porcine vaccines which prevent *T. solium* infection in the pig may however provide a more effective model of prevention when used in addition to mass chemotherapy.

At present the most successful candidate vaccine is Tso18 that has been used to prevent experimental challenge of *T. solium* in pigs. This candidate however has not been tested in larger scale field trials nor produced on an industrial level. Trials for this purpose are in process.

There is therefore a need to have a group of candidate vaccine available. In this study we examined whether a recently derived novel recombinant protein would be a suitable vaccine candidate in a porcine *T. solium* model.

The previous method for obtaining Tso18 was to use homology cloning of a gene that had demonstrated vaccine effectivity against *Taenia ovis* [28]. In this study we used a different approach by taking a protein band that was associated with immune protection in pigs and then defining that band in 2D gel electrophoresis. The spots were then assayed for peptides by mass spectroscopy and the gene pattern determined by RACE PCR.

This protein is important as part of the genomic sequence of *T. solium* and has been used for detection of tapeworm eggs. This study reports that it will not however be a useful vaccine since its level of protection is poor in comparison to the Tso18 and Tso145 candidates. In the future we hope to model differences in structure in order to improve upon the vaccination efficacy of Tso31.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Garcia HH, Del Brutto OH, Nash TE, White AC Jr, Tsang VC, Gilman RH. New concepts in the diagnosis and management of neurocysticercosis (*Taenia solium*). *Am J Trop Med Hyg* 2005;72:3–9. [PubMed: 15728858]
2. Garcia HH, Del Brutto OH. Cysticercosis Working Group in Peru. Neurocysticercosis: updated concepts about an old disease. *Lancet Neurol* 2005;4:653–61. [PubMed: 16168934]
3. Bern C, Garcia HH, Evans C, Gonzalez AE, Verastegui M, Tsang VC, Gilman RH. Magnitude of the disease burden from neurocysticercosis in a developing country. *Clin Infect Dis* 1999;29:1203–9. [PubMed: 10524964]
4. Garcia HH, Gilman R, Martinez M, Tsang VC, Pilcher JB, Herrera G, Diaz F, Alvarado M, Miranda E. Cysticercosis as a major cause of epilepsy in Peru. The Cysticercosis Working Group in Peru (CWG). *Lancet* 1993;341:197–200. [PubMed: 8093496]
5. Palacio LG, Jimenez I, Garcia HH, Jimenez ME, Sanchez JL, Noh J, Ahn L, Mora O, Giraldo M, Tsang VC. Neurocysticercosis in persons with epilepsy in Medellin, Colombia. The Neuroepidemiological Research Group of Antioquia. *Epilepsia* 1998;39:1334–9. [PubMed: 9860070]
6. Gonzalez AE, Cama V, Gilman RH, Tsang VC, Pilcher JB, Chavera A, Castro M, Montenegro T, Verastegui M, Miranda E, Bazalar H. Prevalence and comparison of serologic assays, necropsy, and tongue examination for the diagnosis of porcine cysticercosis in Peru. *Am J Trop Med Hyg* 1990;43:194–9. [PubMed: 2389823]
7. Onah DN, Chiejina SN. *Taenia solium* cysticercosis and human taeniasis in the Nsukka area of Enugu State, Nigeria. *Ann Trop Med Parasitol* 1995;89:399–407. [PubMed: 7487226]
8. Gilman, RH.; Garcia, HH.; Gonzalez, AE., et al. Short cuts to development: methods to control the transmission of cysticercosis in developing countries. In: Garcia, HH.; Marquez, SM., editors. *Taenia solium taeniasis/cysticercosis*. Lima: Editorial Universo; 1999. p. 313–26.
9. Schantz, PM. *Taenia solium* cysticercosis/taeniosis a potentially eradicable disease: developing a strategy for action and obstacles to overcome. In: Garcia, HH.; Marquez, SM., editors. *Taenia solium taeniasis/cysticercosis*. Lima: Editorial Universo; 1999. p. 215–18.
10. Patak KML, Gaur SNS. Immunization of pigs with culture antigens of *Taenia solium*. *Vet Parasitol* 1990;34:353–357. [PubMed: 2316180]
11. Plancarte A, Flisser A, Gauci CG, Lightowlers MW. Vaccination against *Taenia solium* cysticercosis in pigs using native and recombinant oncosphere antigens. *Int J Parasitol* 1999;29:643–7. [PubMed: 10428641]
12. Verastegui M, Gilman RH, Gonzalez A, García HH, Gavidia C, Falcon N, Bernal T, Arana Y, Tsang VC. Cysticercosis Working Group In Peru. *Taenia solium* oncosphere antigens induce immunity in pigs against experimental cysticercosis. *Vet Parasitol* 2002;108:49–62. [PubMed: 12191899]
13. Lightowlers MW, Gauci CG, Chow C, Drew DR, Gauci SM, Heath DD, Jackson DC, Dadley-Moore DL, Read AJ. Molecular and genetic characterisation of the host-protective oncosphere antigens of taeniid cestode parasites. *Int J Parasitol* 2003;33:1207–17. [PubMed: 13678636]
14. Flisser A, Gauci CG, Zoli A, Martinez-Ocana J, Garza-Rodriguez A, Dominguez-Alpizar JL, Maravilla P, Rodriguez-Canul R, Avila G, Aguilar-Vega L, Kyngdon C, Geerts S, Lightowlers MW. Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infect Immun* 2004;72:5292–7. [PubMed: 15322025]

15. Gonzalez AE, Gauci CG, Barber D, Gilman RH, Tsang VC, Garcia HH, Verastegui M, Lightowlers MW. Vaccination of pigs to control human neurocysticercosis. *Am J Trop Med Hyg* 2005;72:837–9. [PubMed: 15964973]
16. Jeri C, Gilman RH, Lescano AG, Mayta H, Ramirez ME, Gonzalez AE, Nazerali R, Garcia HH. Species identification after treatment for human taeniasis. *Lancet* 2004;363:949–50. [PubMed: 15043964]
17. Verastegui M, Gilman RH, Garcia HH, Gonzalez AE, Arana Y, Jeri C, Tuero I, Gavidia CM, Levine M, Tsang VC. Cysticercosis Working Group in Peru. Prevalence of antibodies to unique *Taenia solium* oncosphere antigens in taeniasis and human and porcine cysticercosis. *Am J Trop Med Hyg* 2003;69:438–44. [PubMed: 14640505]
18. Mayta H, Talley A, Gilman RH, Jimenez J, Verastegui M, Ruiz M, Garcia HH, Gonzalez AE. Differentiating *Taenia solium* and *Taenia saginata* infections by simple hematoxylin-eosin staining and PCR-restriction enzyme analysis. *J Clin Microbiol* 2000;38:133–7. [PubMed: 10618076]
19. Stevenson P. Observations on the hatching and activation of fresh *Taenia saginata* eggs. *Ann Trop Med Parasitol* 1983;77:399–404. [PubMed: 6685462]
20. Tsang VC, Peralta JM, Simons AR. Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying the specificities of antigens and antibodies separated by gel electrophoresis. *Methods Enzymol* 1983;92:377–91. [PubMed: 6855619]
21. Tsang VC, Brand JA, Boyer AE. An enzyme-linked immunoelectrotransfer blot assay and glycoprotein antigens for diagnosing human cysticercosis (*Taenia solium*). *J Infect Dis* 1989;159:50–59. [PubMed: 2909643]
22. Chittum HS, Lane WS, Carlson BA, Roller PP, Lung FT, Lee BJ, Hatfield DL. Rabbit b-globin is extended beyond its UGA stop codon by multiple suppressions and translational reading gaps. *Biochemistry* 1998;37:10866–10870. [PubMed: 9692979]
23. Waterkeyn JG, Gauci C, Cowman AF, Lightowlers MW. Codon usage in *Taenia* species. *Exp Parasitol* 1998;88:76–8. [PubMed: 9501853]
24. Ferrer E, Gonzalez LM, Martinez-Escribano JA, Gonzalez-Barderas ME, Cortez MM, Davila I, Harrison LJ, Parkhouse RM, Garate T. Evaluation of recombinant HP6-Tsag, an 18 kDa *Taenia saginata* oncospherical adhesion protein, for the diagnosis of cysticercosis. *Parasitol Res.* 2007 Mar 12;[Epub ahead of print]
25. Green MR. Pre-mRNA splicing. *Annu Rev Genet* 1986;20:671–708. [PubMed: 2880558]
26. Gauci CG, Lightowlers MW. Alternative splicing and sequence diversity of transcripts from the oncosphere stage of *Taenia solium* with homology to the 45W antigen of *Taenia ovis*. *Mol Biochem Parasitol* 2001;112:173–81. [PubMed: 11223124]
27. Gauci CG, Flisser A, Lightowlers MW. A *Taenia solium* oncosphere protein homologous to host-protective *Taenia ovis* and *Taenia saginata* 18 kDa antigens. *Int J Parasitol* 1998;28:757–60. [PubMed: 9650055]
28. Gauci C, Lightowlers MW. Molecular cloning of genes encoding oncosphere proteins reveals conservation of modular protein structure in cestode antigens. *Mol Biochem Parasitol* 2003;127:193–8. [PubMed: 12672528]
29. Benitez L, Garate T, Harrison LJ, Kirkham P, Brookes SM, Parkhouse RM. Cloning and sequencing of the gene encoding the principal 18-kDa secreted antigen of activated oncospheres of *Taenia saginata*. *Mol Biochem Parasitol* 1996;78:265–8. [PubMed: 8813696]
30. Alderete JF, Benchimol M, Leher MW, Crouch ML. The complex fibronectin--*Trichomonas vaginalis* interactions and Trichomonosis. *Parasitol Int* 2002;51:285–92. [PubMed: 12243782]
31. Calvet CM, Meuser M, Almeida D, Meirelles MN, Pereira MC. *Trypanosoma cruzi*-cardiomyocyte interaction: role of fibronectin in the recognition process and extracellular matrix expression in vitro and in vivo. *Exp Parasitol* 2004;107:20–30. [PubMed: 15208034]
32. Schwarz-Linek U, Hook M, Potts JR. The molecular basis of fibronectin-mediated bacterial adherence to host cells. *Mol Microbiol* 2004;52:631–41. [PubMed: 15101971]
33. Hammerschmidt S. Adherence molecules of pathogenic pneumococci. *Curr Opin Microbiol* 2006;9:12–20. [PubMed: 16338163]

34. Kyngdon CT, Gauci CG, Gonzalez AE, Flisser A, Zoli A, Read AJ, Martinez-Ocana J, Strugnell RA, Lightowlers MW. Antibody responses and epitope specificities to the *Taenia solium* cysticercosis vaccines TSOL18 and TSOL45-1A. *Parasite Immunol* 2006;28:191–9. [PubMed: 16629704]
35. Gauci CG, Verastegui MR, Gilman RH, Lightowlers MW. *Taenia solium* and *Taenia ovis*: Stage-specific expression of the vaccine antigen genes, TSOL18, TSOL16, and homologues, in oncospheres. *Exp Parasitol* 2006;113:272–5. [PubMed: 16510142]
36. Waterkeyn J, Gauci C, Cowman A, Lightowlers M. Sequence analysis of a gene family encoding *Taenia ovis* vaccine antigens expressed during embryogenesis of eggs. *Mol Biochem Parasitol* 1997;86:75–84. [PubMed: 9178269]

Figure 1.

Colloidal Coomassie Blue stained oncosphere proteins on two-dimensional gel.

First dimension IEF was carried out at pH 4–7, second dimension gel electrophoresis was performed using a 5–22.5% gradient SDS gel. Three Tso31 specific spots are shown: OA1, OA2, and OA3. The left lane on the gel was loaded with 0.1 µg/mm of crude oncosphere antigen. Before staining the left part of the gel was cut, blotted and probed using sera from pigs vaccinated with oncosphere crude extracts, which were protected against cysticercosis.

Figure 2.

Antibody characterization of Tso31 protein.

Oncosphere (lane 1–3), cyst (lane 4) and adult tapeworm (lane 5) crude antigens were SDS gel electrophoresed (5–22.5% gradient SDS gel) and blotted. Lane 1 was probed with sera from pigs vaccinated with crude oncosphere antigen protected against cysiticercosis; lane 2 was probed with sera from pre-immunized rabbits; and lanes 3–5 were probed with rabbit sera immunized with rGST-Tso31 protein respectively.

Figure 3.

Indirect immunofluorescence (IFI) of oncospheres.

Oncospheres were IFI stained using sera from rabbits immunized with rGST-Tso31 and fluorescein labeled goat anti-rabbit antibody (see text for more details). Oncospheres were permeabilized using Triton X-100 (upper panel) or directly stained (non permeabilized lower panel). Stained oncospheres were then observed under normal light were (panels a and c) and UV light (panels b and d) under 1000X magnification. Only fully activated oncospheres are shown in the lower panel. An arrow indicates a fully activated oncosphere in the upper panel.

Figure 4.

Antibody response to the recombinant GST-Tso31 protein.

The curves represent the mean antibody titers of the eight vaccine pigs and the four pigs in the control group respectively. Sera from pigs were diluted 1:6400 in PBS. No differences on the OD values was observed in the antibody response among the vaccinated pigs or among control pigs ($P > 0.05$, Mann Whitney test). But, except for the pre-immune sera, the immune response to the GST-Tso31 protein was different when vaccinated group were compared to the control group ($P = 0.003$, Mann Whitney test). Arrow heads indicate time of challenge. The bars represent the 95 percentile of the median values.

Table 1

Numbers of *Taenia solium* cysticerci in vaccinated and control pigs

Group	Tapeworm ID *	Cyst count		Total	Total median (95% Conf. Interval)
		Viable	Degenerate		
Control					
rGST	1	1825	49	1874	
rGST	3	2793	29	2822	
rGST	2	2002	936	2938	2348
rGST	2	224	400	624	(624 – 2938)
Vaccinated					
rGST-Tso31	2	0	782	782	
rGST-Tso31	2	0	0	0	
rGST-Tso31	3	41	358	399	
rGST-Tso31	1	2175	95	2270	
rGST-Tso31	1	1404	290	1694	861
rGST-Tso31	1	1011	122	1133	(269 – 1881)
rGST-Tso31	1	753	186	939	
rGST-Tso31	1	472	112	584	

* Proglotids from three different tapeworms were used for infection.