Peptide epitopes of the *Taenia solium* antigen Ts8B2 are immunodominant in human and porcine cysticercosis

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**A B S T R A C T**

Ts8B2 is a gene which encodes for a member of the *Taenia solium* metacestode 8 kDa antigen family. Since the Ts8B2-GST recombinant protein compares very favourably with other diagnostic antigens, and in order to study the antigenic nature and structure of this molecule, the Ts8B2 was expressed in prokaryotic and eukaryotic systems. The diagnostic potential of the recombinant Ts8B2 proteins was evaluated by enzyme-linked immunosorbent assays (ELISA) using a collection of serum and cerebrospinal fluid (CSF) samples from patients with clinically defined neurocysticercosis (NCC), and also sera from *T. solium* infected pigs. Despite the predicted glycosylation of the Ts8B2-Bac recombinant protein, there was very little difference in assay sensitivity/specificity when the Ts8B2 reagent was expressed in either prokaryotic or eukaryotic systems, suggesting that peptidic Ts8B2 epitopes are immunodominant in porcine cysticercosis and human neurocysticercosis. Conveniently, production of recombinant Ts8B2 in *Escherichia coli* is economical and facile, making it a feasible and practical choice as a diagnostic reagent for use in endemic areas. The Ts8B2 ELISA is particularly useful for the diagnosis of active as opposed to inactive cases of NCC and conduct of the assay is also facilitated by the fact that assay sensitivity is significantly greater when serum as opposed to CSF samples are employed.

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Neurocysticercosis (NCC) is the most common helminth disease of the human nervous system and is caused by infection with metacestode larvae of the tapeworm *Taenia solium*. This disease is a public health and economic problem in many developing countries of Latin America, Asia and Africa, but has also been reported in the United States of America and in several European countries [1,2]. Specific and sensitive diagnosis is a prerequisite for accurate clinical diagnosis and for evaluating disease endemicity, before, during and after the application of control programs. Ts8B2 is an 8 kDa putative secreted *T. solium* metacestode antigen. It has previously been bacterially expressed as a recombinant glutathione S-transferase (GST) fusion protein (Ts8B2-GST) and used in an enzyme-linked immunosorbent assay (ELISA). This Ts8B2 ELISA assay exhibits high specificity and sensitivity, particularly for the diagnosis of active as opposed to inactive cases of NCC, and has the advantage that assay sensitivity is greater when serum as opposed to CSF samples are employed [3]. In order to determine whether the GST molecule influences the sensitivity and specificity of the ELISA assay, we have now similarly evaluated Ts8B2, expressed as a histidine fusion protein (Ts8B2-His). Furthermore, since expression of recombinant antigens in prokaryotic systems can be associated with inadequate post-translation modifications, such as folding and glycosylation, and since N-linked carbohydrates are possibly

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**Abbreviations:** NCC, neurocysticercosis; CNS, central nervous system; CSF, cerebrospinal fluid; E/S, excretory/secretory antigens; ELISA, enzyme-linked immunosorbent assay.
of importance in the antigenicity of

T. solium metacestode glyco-proteins [4], we also evaluated Ts8B2 expressed in the eukaryotic Baculovirus system, where folding, glycosylation, phosphorylation and oligomerization occur in a similar manner to that of mammalian cells [5,6]. These 3 recombinant antigens were tested in ELISA assays with serum and cerebrospinal fluid (CSF) from clinically defined human cases of neurocysticercosis (NCC) and also with serum samples from T. solium infected pigs. The serum and CSF samples, and the cloning and expression of GST-Ts8B2 have been previously described [3]. As an additional antigenic target for the ELISA assays, vesicular fluid was collected from metacestodes of T. solium from infected pigs [7]. Briefly, T. solium vesicular fluid shows an excellent sensitivity although its poor specificity hampers its use in the disease diagnosis [3].

The histidine fusion protein Ts8B2-His was obtained by sub-cloning the Ts8B2 coding sequence into the expression vector pQE30 (Qiagen, Chatsworth, CA), which adds a polyhistidine tag to the N terminus of the recombinant polypeptide. The resulting recombinant plasmid was used to transform M15 strain E. coli cells (Qiagen), and expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, Madrid, Spain).

For expression in Baculovirus, first, an N-terminal polyhistidine tag was introduced into the vector pBacPAK 9 (Clontech, Mountain View, CA). An EcoRI–KpnI restriction fragment of the Ts8B2 CDNA coding sequence from the recombinant plasmid pQE50 was cloned into the same restriction sites of the Baculovirus transfer vector pBacPAK 9 (Clontech). The recombinant viral expression vector was generated by cotransfection of the Sf9 (Spodoptera frugiperda) insect ovarian cell line, with Bsu36I-digested BacPAK6 viral DNA (Clontech), and the recombinant transfer vector pBacPAK 9 containing the Ts8B2 gene. The recombinant virus was verified by PCR using Bac1 and Bac2 primers (Clontech) and then sequenced. The Sf9 insect ovarian cell line was cultured at 27 °C in serum-free medium Insect-X-Press (BioWhittaker). High-titre recombinant Baculovirus stock (10⁶ plaque forming unit/ml; pfu/ml) was used to infect Sf9 cells at a multiplicity of infection of 10. The infected Sf9 cells were cultured at 27 °C and collected for assays when cytopathic effect was evident (around 72 h post-infection). Although cell lines from S. frugiperda SF-9 and SF-21 are commonly used for Baculovirus propagation “in vitro”, their larvae are cannibalistic, and so, the larvae of Trichoplusia ni are preferred for expression “in vivo” as they are easy to handled in large numbers and express high levels of recombinant protein by 2–3 days after infection [6]. The T. ni eggs were maintained in larva developmental cages, containing artificial insect diet [6]. After hatching, and 10–14 days at 24 ± 1 °C in a climatic chamber, the larvae grew to about 3 cm in length. Fourth instar larvae were sedated by incubation on ice for 15 min and then injected near the proleg (forward along the body cavity) with 20 μl of medium containing recombinant Baculovirus. When the larvae became pale, swollen and lethargic (72–96 h after infection), they were harvested and frozen immediately at −70 °C.

Recombinant proteins were prepared from both the insect cell cultures and the Baculovirus infected larvae. Baculovirus infected cells were lysed by hypotonic shock treatment with 25 mM NaHCO₃ pH 8.3. Cell debris were removed by centrifugation and a 1/10 volume of 10 × PBS was added. Infected larvae were homogenized on ice with a tissue disruptor in the presence of 0.75 ml/larva of extraction buffer (2.5 mM dithiothreitol, 0.01% Triton X-100, 1 mM phenylmethylsulfonyl fluoride in phosphate buffered saline). The resulting crude extract was centrifuged at 12,000 rpm for 30 min. The supernatant was filtered and used to purify the recombinant protein.

For purification of recombinant proteins, bacterial cultures were lysed using B-PE® Bacterial Protein Extractor Reagent (Pierce, Cheshire, UK). Expressed His-fusion protein was purified from the lysate by affinity chromatography with Ni-NTA Spin kit as described by the manufacturer (Qiagen), and GST recombinant protein as reported before [4]. Recombinant protein was similarly purified from Baculovirus infected cells and larvae using Ni-NTA Spin (Qiagen). The purified proteins were checked by SDS-PAGE. The bacterially expressed Ts8B2-His had the predicted molecular mass of 11 kDa (10 kDa of Ts8B2, plus 1 kDa of histidine tag). In contrast, the full length Ts8B2-Bac recombinant protein expressed in either Sf9 cells or T. ni larvae was 14 kDa, 3 kDa higher than the size predicted by the deduced amino acid sequence of Ts8B2 plus the histidine tag, a difference possibly accounted for the predicted N-glycosylation site in the carboxy-terminal part of the molecule.

Purified Ts8B2-GST, Ts8B2-His, Ts8B2-Bac and cyst fluid based ELISA were performed using the following, previously described, samples [3,9]:

- 62 serum samples from patients with NCC which had been confirmed on the basis of symptomology and neuro-imaging consisting of 31 samples from active NCC cases and 31 samples from inactive NCC cases. All of these cases had cysticerci located in the brain parenchyma. Paired CSF samples were available from 26 of the cases with active NCC and 23 of the cases with inactive NCC.
- 138 serum samples from cases of ‘probable’ NCC from endemic areas of Latin America. Diagnosis was based on symptomology alone as there was no access to neuro-imaging facilities.
- 57 serum samples from individuals with other clinically defined helminth infections [3] and 30 serum samples from healthy volunteers were used to determine assay specificity.
- 20 CSF samples from patients with other neurological disorders.
- 8 porcine serum samples from naturally T. solium-infected pigs from an endemic area in Mexico and 5 serum samples from pigs from a T. solium free intensive pig production unit in Mexico were used as negative controls [3,10,11].

The ELISA results for the prokaryotic and eukaryotic recombinant Ts8B2 proteins were similar and comparable to those described previously for Ts8B2-GST [3]. Examination of the results for the paired serum and CSF samples confirmed that assay sensitivity was considerably greater when serum samples were employed as opposed to CSF. If a CSF sample was positive the corresponding paired serum sample was also positive and this held true with all 3 ELISA assays. Assay sensitivity was also consistently greater for cases of active as opposed to inactive NCC. Combining the results for the serum samples for the paired and single samples indicated the overall sensitivity of the Ts8B2-GST ELISA was 96.8% and 67.7% for active and inactive NCC, respectively (Table 1). As previously reported [3], the 138 patients with a diagnosis of probable cysticercosis exhibited similar results to those with confirmed inactive NCC (data not shown). In the porcine system, assay sensitivity 6/8 (75%) was identical for all three Ts8B2 recombinant protein based ELISA’s. Finally there was no difference in the sensitivity/specificity of Ts8B2-Bac based ELISA’s employing recombinant proteins purified from Sf9 cells or T. ni larvae (data not shown).

The similar performance of prokaryote and eukaryote expressed Ts8B2 in the ELISA assay suggests that the immunodominant epitopes of Ts8B2 are predominantly peptidic. In practical terms, this may be considered advantageous as the production of the recombinant antigen in prokaryotes is simple and cheap, and thus feasible in developing countries. In addition, the similar results obtained with the GST and His-fusion proteins indicate that neither GST nor the His tag interfere with the antigenicity of the Ts8B2 molecule. Thus data presented here confirms and extends our earlier observation [3] that the excreted/secreted Ts8B2 antigen of T. solium metacestodes has promise for the diagnosis of active NCC and, perhaps, porcine cysticercosis. The sensitivity for inactive NCC, although
lower (67.7%) is still an improvement over the generally low sensitivity previously reported [10].

In contrast to Ts8B2, another Taenia spp. antigen (HP6-Tsag) was more sensitive for the detection of inactive NCC, when expressed in the eukaryotic Baculovirus rather than as the prokaryotic recombinant antigen HP6-Tsag-GST. Therefore glycosylation clearly plays a part in the immunogenicity of HP6-Tsag [9]. Baculovirus expressed TsM, another member of the T. solium metacestode 8 kDa diagnostic antigen family, and other glycoprotein antigens such as GP50 and T24, were shown to have improved assay specificity [10–13]. However, no comparison of the performance of any of these antigens expressed in both prokaryotic and Baculovirus expression systems, has been reported.

Not all Baculovirus systems are necessarily similar. For example, the GP50 recombinant protein expressed in the High Five insect cell line was less sensitive in ELISA assay than the protein recovered from the Sf9 cell line, a result attributed to variations in glycosylation between the two systems [14]. Although the High Five insect cells are the same larval species (T. ni) that we used to express Ts8B2-Bac, no such differences in assay performance were noted between the two Ts8B2-Bac recombinant proteins based ELISAs (Table 1). Thus possible differences in glycosylation between these two Baculovirus expression systems apparently have little or no effect on the Ts8B2 serological epitopes recognized in cysticercosis, once again suggesting that the predominant Ts8B2 antigenic epitopes recognised in porcine cysticercosis and human NCC are peptidic rather than glycosidic or conformational. The fact that many bacterially expressed recombinant antigens [3,15–16] and synthetic peptides [17–19] have been used as diagnostic targets for cysticercosis is consistent with this conclusion. Indeed, the recent application of completely synthetic versions of several members of the T. solium metacestode 8 kDa diagnostic antigen family for diagnosis [18,19] also supports this conclusion, although these long synthetic peptides are very expensive, whereas production of prokaryotic recombinant antigen [3,7,10,15,16] and short synthetic peptides [17] is considerably more economical and, importantly, feasible in almost any laboratory. A logical extension of this work would be to determine Ts8B2 immunodominant epitopes by bioinformatic programs (Protein, DNASTar, Lasergene, Madison, USA) and test short overlapping synthetic peptides to select the best one(s) for its (their) use in ELISA.

A comparison of Ts8B2 with other putative diagnosis recombinant antigens reveals its superior diagnostic capability in certain clinical scenarios, an observation that emphasises the paramount importance of using samples from clinically defined cases of NCC when validating diagnostic assays [8,11,17–19]. Thus, the Ts8B2 assay sensitivity was typically higher in cases of active as opposed to inactive cysticercosis, as has been described for other antigens [4,8,11,13,15,20]. This finding raises the interesting possibility of distinct parasite antigens being differentially applicable depending on the stage of evolution of this pleomorphic disease, for example, active versus inactive NCC. The Ts8B2 ELISA assay yielded similar sensitivities for cases defined as probable NCC (63.8–64.5%; data not shown) and for cases of clinically defined inactive NCC (67.7–70.1%). The former cases (138 Venezuelan serum samples) were from patients living in rural endemic areas, but without confirmatory neuro-imaging studies, and diagnosed on clinical grounds as probable NCC. The fact that 90 of these 138 patient sera recognised Ts8B2 raises the possibility that these infections have evolved into a chronic state (inactive NCC), associated with cyst death, calcification, and lower titres of antibodies. As previously reported by us [3] and worth re-emphasising, the sensitivity of the Ts8B2 based ELISA is significantly higher with serum samples than with CSF (P < 0.001). This is fortunate, as serum collection is much less difficult and invasive than the collection of CSF. Other authors, using different systems [10,11,20], have also noted that for diagnosis of NCC, using serum samples can give preferable or comparable sensitivities to that obtained with CSF samples. In conclusion the Ts8B2 based ELISA merits further exhaustive comparative evaluation for the diagnosis of human NCC, and perhaps also porcine cysticercosis.

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