SEROLOGIC DIAGNOSIS OF NEUROCYSTICERCOSIS USING SYNTHETIC 8-KD PROTEINS: COMPARISON OF ASSAY FORMATS

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Abstract. The assay of choice for serological detection of cysticercosis in humans and pigs is the enzyme-linked immunoelectrotransfer blot (EITB), a Western blot assay that relies on the use of seven lentil-lectin–purified glycoproteins (LLGPs) derived from Taenia solium metacestodes. The EITB is has a sensitivity of 98% and a specificity of 100% in detecting T. solium infection in humans and pigs. Four chemically synthesized LLGP antigens, TS14, TS18var1, TSRS1, and TSRS2var1, were assayed individually by enzyme-linked immunosorbent assay (ELISA) and Western blot for immunoreactivity against a large cohort of sera from clinically defined neurocysticercosis patients. The sensitivity and specificity of all four of these antigens using the EITB were well below the standards set by the LLGP EITB, whereas results of the Western blot format closely mirrored those of the LLGP EITB.

INTRODUCTION

The World Health Organization has recently classified neurocysticercosis (NCC) as the most important neurologic disease of parasitic origin in humans. Neurocysticercosis is a major cause of adult-onset epilepsy in areas where the pork tapeworm Taenia solium is endemic. The disease develops after ingestion of tapeworm eggs and the subsequent encystment of the larval metacestode in the tissues of the brain and central nervous system. The cysts can remain viable for many years, but eventually degenerate into inactive granulomas, and finally calcified lesions. Neurocysticercosis can be asymptomatic, but may produce a broad range of clinical manifestations, including headaches, seizures, hydrocephaly, and death. Unfortunately, T. solium-endemic areas are often rural and impoverished, lacking in basic sanitation and adequate medical care. The disease is first detected when infected persons exhibit neurologic symptoms and seek medical treatment in city clinics. Diagnosis of NCC in these cases is performed by computed tomography (CT) or magnetic resonance imaging (MRI) scans of the brain, followed by a confirmatory serologic assay. The enzyme-linked immunoelectrotransfer blot (EITB), a Western blot assay developed in 1989 that has a sensitivity of 98% and a specificity of 100% in detecting pathologically confirmed cases of NCC, was recognized as the serologic assay of choice for the detection of cysticercosis in humans and pigs by the World Health Organization/Pan American Health Organization in 1995. It produces the assay, cysts are excised from porcine tissue, homogenized, and partially purified on a lentil-lectin column. The resulting lentil-lectin glycoproteins (LLGPs) are then separated by electrophoresis and blotted onto a nitrocellulose matrix for detection with NCC-specific patient antibody. Separation of LLGP results in seven distinct antigenic bands of 13, 14, 18, 21, 24, 39–42, and 50 kD (Figure 1A). Antibody reactivity with any one of these bands indicates exposure to cysticercosis.

Although the EITB has been instrumental in detecting NCC where brain imaging equipment and expertise is unavailable, its dependence on the acquisition and purification of native cyst antigen make it impractical and costly. Furthermore, the EITB is not quantitative. A quantitative assay that detects antibodies to cysticercosis may provide a means to monitor patient disease status and efficacy of chemotherapy. One quantitative assay format, the enzyme-linked immunosorbent assay (ELISA), performed in polystyrene plates, is particularly desirable, because it lends itself to high-throughput diagnostic applications. Previous attempts to use LLGP in an ELISA format have failed because LLGP contains irrelevant proteins that bind to polystyrene with a higher affinity than any of the seven T. solium-specific antigens. To build a new assay format that maintained the sensitivity and specificity of the EITB without its reliance on native antigen, we isolated the seven diagnostic proteins in LLGP, cloned and sequenced them, then reproduced them either synthetically or recombinantly. The LLGP antigens of 13, 14, 18, and 21-kD (Figure 1A) have been identified as belonging to a large, closely related group of proteins representing four distinct phylogenetic clades, the 8-kD antigen family.
MATERIALS AND METHODS

**Defined serum collections.** Defined cysticercosis serum samples (n = 377) were collected at the Nacional de Ciencias Neurologicas (Lima, Peru) from patients presenting with clinical symptoms of NCC. Patients were confirmed as having NCC by both EITB serology and CT or MRI brain imaging as outlined by del Brutto and others. Patient samples were collected in compliance with protocols reviewed and approved by the ethical review boards of all institutions concerned (Centers for Disease Control and Prevention and Universidad Peruana Cayetano Heredia). All patients involved in this study provided written informed consent. Patients were assorted according to the presence and number of viable, racemose (malignant), degenerating, and calcified cysts shown by brain images. Serum from patients diagnosed as having either ≥ 2 viable cysts or a racemose cyst (n = 107) in this collection were used to determine the overall antigen sensitivity in both assay formats because single lesions are not clear indicators of NCC. Antigen-specific sensitivity for serum antibodies from patients diagnosed as having only single, viable cysts (n = 20), degenerating (dying) cysts (n = 66), and inactive, calcified cysts (n = 114) was also evaluated. Brain scans of many patients (n = 70) showed cysts in several stages of viability, and were evaluated separately. To define the specificity of the 8-kD antigens for cysticercosis, serum samples from healthy individuals who had not traveled outside the United States (normal human sera [NHS]) (n = 146), and sera from persons infected with other parasitic diseases from countries where NCC is not endemic (n = 162) were tested. The infected serum used to test for specificity were from confirmed cases of infection with *Ascaris lumbricoides* (6), *Cryptosporidium parvum* (10), *Echinococcus granulosus* (20), *Echinococcus multilocularis* (1), *Fasciola hepatica* (4), *Leishmania donovani* (1), *Leishmania tropica* (1) *Plasmodium falciparum* (16), *Schistosoma haematobium* (17), *Schis-

tosoma mansoni* (36), *Trichinella spp.* (16), and *Taenia saginata* (14). Normal human sera (20) from an area in Egypt that is socioeconomically similar to Peru, as well as highly endemic for exposure to schistosomiasis and other parasitic diseases, were also included for their potential cross-reactivity.

**8-kD antigens.** The full-length, 8-kD proteins were chemically synthesized without their signal sequences by commercial manufacturers at a purity of ≥ 95% as follows: TS14 (AF082829), (Grýphon Sciences, San Francisco, CA and AnaSpec Inc., San Jose, CA); TS18var1 (AF098073), (AnaSpec); TSRS1 (AF082830) (AnaSpec); and TSRS2var1 (AF356343), (SynPep Corp., Dublin, CA and AnaSpec). All of the synthetic 8-kD mature protein sequences are 66 amino acids in length, with the exception of TS18var1, which is 67 amino acids long (Table 1). In cases where individual proteins were acquired by more than one manufacturer, intra-assay comparisons of specific activity showed that there was no difference in protein performance between manufacturers.

**Enzyme-linked immunosorbent assay.** The quantitative determination of patient seroreactivity to the 8-kD proteins was determined by the Falcon® assay screening test (FAST)–ELISA method described previously. Briefly, iophosphoryl 8-kD antigens were solubilized in 0.05 M N-2-hydroxyethylpiperazine-N’-ethane sulfonic acid (HEPES), pH 7.0, 0.1 M NaCl, 2 mM dithiothreitol (DTT) and quantitated by 280 nM ultraviolet (UV) spectrophotometry, using the extinction coefficients of each. To prevent freezing at the storage temperature of −80°C, glycerol was added to a final concentration of 43.5%. All antigens were assayed separately in excess. Each was diluted in phosphate-buffered saline (PBS), pH 7.2, to a concentration of 0.01 mg/mL, then sensitized to the polystyrene stcks of NUNC-TSP transferable solid-phase screening system lids (catalog no. 445497; Nalge Nunc International, Roskilde, Denmark) from the corresponding 96-well flat bottom incubation plates (catalog no. 269620; Nalge Nunc

![Figure 1](image-url)
Table 1

<table>
<thead>
<tr>
<th>Protein name</th>
<th>GenBank accession no.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TS14</td>
<td>AF082629</td>
<td>EKKNPKDVANSTKGIEVYHEHFEHDPIGKQAQLAKEWKEAMLDEGKVKRTSLVEHCGBPQKKTTA</td>
</tr>
<tr>
<td>TS18var1</td>
<td>AF098073</td>
<td>EEKIPKCDTSTKKEIEYHWNWFFDPIDPGKQAQLAKWNTEVQGAKKEIRAELAYCRLKGAATTA</td>
</tr>
<tr>
<td>TSRS1</td>
<td>AF082830</td>
<td>EETKPCDVVKNKKGMEVYKFFYEDPLGKIAQLAKDWKAMELARSKVRASLAEYIRGLNKEAA</td>
</tr>
<tr>
<td>TSRS2var1</td>
<td>AF356343</td>
<td>EKNKTDDVKGSKWNIEFVHRRFYEDPGKQAQLAKDNAETAP pensions CRVRLAENRRGLKNTA</td>
</tr>
</tbody>
</table>

International. The sticks were sensitized for two or more hours at room temperature with shaking.

**Human IgG standard curve.** The internal standard curve used in our FAST-ELISA method is ordinarily comprised of pooled, cysticeriosis-positive patient serum titrated to represent 10 unit values of seroreactivity. When tested against the 8-kD antigens, the seroreactivity of the pooled serum used in the standard curve was found to vary in intensity between antigens.

To create a universal standard that was independent of antigen-specific bias, we constructed a curve using normal human IgG (hIgG). Lyophilized, purified hIgG was rehydrated in PBS, 0.1% sodium azide and quantitated using the Bradford protein assay. The solubilized hIgG was used to construct a 10-point standard curve by dilution into PBS, 0.01% bovine serum albumin factor V, 0.1% sodium azide with IgG concentrations ranging form 0.01 μg/mL to 1 μg/mL. The standards were assigned unit values (from 0.01 to 1) based on their respective concentrations and all subsequent ELISAs were measured on this activity scale. The standard curve was dispensed into empty wells of the plate and sensitized to the polystyrene sticks at the same time the antigens were applied. In place of test sera, antibody dilution buffer was added to these wells during the sera incubation step.

**Enzyme-linked immunosorbent assay format.** After sensitization with antigen and the standard curve, the plates were rinsed using a wash buffer of PBS, 0.3% Tween 20 (polyoxyethylene sorbitan monolaureate; Calbiochem, San Diego, CA) using a pressurized garden sprayer. Washes were performed in this manner between all subsequent reagent incubation steps. All reagents were dispensed at a concentration of 150 μL/well, and incubated at room temperature for five minutes with constant shaking. All serum samples were diluted 1:100 in PBS, 0.3% Tween 20, 5% nonfat dry milk and tested in triplicate. Appropriate negative and positive controls were used on each plate to ensure intra-assay integrity. Goat anti-human heavy and light chain IgG conjugated to horseradish peroxidase (GAHG-POD), 1.86 mg/mL, was diluted to a working concentration of 1:1,000 in wash buffer and used to detect reactive antibody, followed by development with SureBlue™ 3′,3′,5,5′-tetramethylbenzidine (TMB) microwell peroxidase substrate (one component; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD). Absorbance at 650 nm was read with a THERMOmax microplate reader using SoftMax Pro 4.7.1 software (Molecular Devices Corp., Sunnyvale, CA). Inters assay coefficient of variance for the positive controls was calculated to ensure validity of the assay. Plates with a positive control mean unit value ± 2 standard deviations outside the inters assay mean were repeated.

**Enzyme-linked immunoelectrotransfer blot.** For the EITB assay format, TS14, TS18var1, and TSRS2var1 were solubilized in a 50 mM DTT solution of 0.05 M HEPES, 0.1 M NaCl to prevent polymerization via disulfide bonding. Since TSRS1 contains no cysteines, no DTT was included in the solubilization buffer. Proteins were quantitated by UV absorption as described earlier in this report. The EITB was performed as previously described. All defined cysterciosis, other parasitic diseases, and NHS samples were tested for seroreactivity against all four of the 8-kD antigens. Assays for each antigen were repeated by a second party to ensure reproducibility. Serum samples were diluted 1:100 in 0.05 M Tris, 0.5 M NaCl, 0.3% Tween 20, 5% nonfat dry milk and dispensed into disposable incubation trays (catalog no. 1048015; Schleicher and Schuell, Keene, NH). All reagents were dispensed at a volume of 0.7 mL per trough. Nitrocellulose test strips and diluted serum samples were incubated in the trays overnight at 4°C with gentle rocking, then washed four times (five minutes per wash) in wash buffer using the Accutran™ automatic strip washer (Schleicher and Schuell). GAHG-POD conjugate was diluted 1:8,000 in 0.05 M Tris, 0.5 M NaCl, 0.3% Tween 20, and the strips were incubated for one hour at room temperature with gentle rocking. The antigen strips were then washed three times (five minutes per wash) with wash buffer and two times in PBS, before a 10-minute incubation in 3,3′-diaminobenzidine substrate. After substrate development, strips were rinsed 10 times in distilled water, dried, and aligned for analysis.

To determine the best loading concentration for each antigen, all proteins were titrated 1:2 in a range from 0.05 mg/mL to 0.0005 mg/mL and assayed by Western blot against bulk serum samples of known reactivity to the original native-antigen EITB. The bulk sera used were collected from an NCC patient with very high seroreactivity in the EITB, an NCC patient with very low seroreactivity in the EITB, a patient from a country not endemic for cysterciosis infected with a different parasite, and an NHS sample. The ideal gel-loading concentrations were 0.005 mg/mL for TS14, 0.001 mg/mL for TS 18var1, 0.020 mg/mL for TSRS1, and 0.005 mg/mL for TSRS2var1. Pilot studies indicated that the three antigens that contain cysteines (TS14, TS18var1, and TSRS2var1) had to be treated with 50 mM DTT to prevent self-polymerization into higher molecular weight entities. It was also found that these proteins tended to frequently form non-specific protein-protein interactions in the blot format. This necessitated the use of 0.05 M Tris, 0.5 M NaCl as a reagent diluent buffer, which has a higher ionic strength than PBS, the reagent diluent buffer used in the native EITB.

**RESULTS**

**Enzyme-linked immunosorbent assay.** All protein antigens were directly coated to polystyrene at an excess concentration
of 0.01 mg/mL. The initial evaluation study was performed using representative subsets of the Peruvian clinically defined sera (n = 206), NHS (n = 97), and the sera from patients infected with other parasitic diseases from non-endemic countries (n = 79). Sensitivity of each antigen for patient sera in various clinical stages was calculated, as well as the specificity of NCC serum antibody against cysticercosis antigens (Table 2). Overall assay efficacy, as measured by Youden’s J index ([Sensitivity + Specificity] − 1) was calculated using patients showing either ≥ 2 viable cysts or racemose cases of NCC by brain imaging techniques. Antigen sensitivity in detecting seroreactivity of patients with viable single cysts, degenerating cysts, and inactive calcifications is also shown. Patients with mixed infections (n = 41) were excluded from the analyses shown.

Each of the four 8-kD antigens had distinctly different reactivities with all serum samples tested. The cutoff points for each antigen were selected based on the best value obtained using Youden’s J index as a measure of efficiency. Plots showing the performance of all four 8-kD antigens and their respective cutoff values are shown in Figure 2. Since this evaluation study was repeated with highly similar results, we discontinued testing in this assay format.

### Enzyme-linked immunoelectrotransfer blot

With the exception of TS14, all protein antigens performed better in the EITB format than in the ELISA, as shown by increases in Youden’s J index, which was again calculated using only those sera samples from patients with multiple and racemose cysts (n = 107) against the serum from persons with other parasitic diseases from countries not endemic for NCC (n = 162) and NHS samples (n = 146) (Tables 2 and 3). Since the J index values for all four antigens were similar to those shown in Table 3 when calculated using only those NCC and control serum used for evaluation of the 8-kD ELISA (n = 382) (Table 2), the results of the larger cohorts are described. TSRS1 showed the greatest increase in sensitivity to multiple viable and racemose cysts (from 77% to 96%), whereas all other proteins showed lesser change in this disease category (≥ 5%). Likewise, TSRS1 showed the greatest improvement overall in detecting all other disease states, increasing in sensitivity by 35% for single cysts, 28% for degenerating cysts, and 27% for calcified cysts. TS14 also showed an increase in detecting various disease states, but was found to be highly cross-reactive with non-NCC sera, with a specificity of only 76%, which was lower than that of the ELISA format (85%). Except for TS14, all protein antigens showed improvement in specificity. Only TSRS1 improved in specificity for all disease categories, as well as showing increased specificity over the ELISA format.

Both TSRS1 and TS18var1 had Youden’s J index values greater than 0.9 in the EITB format, which is indicative of an efficient assay. Visual comparison of the two antigens on blot run against the same set of NCC serum samples shows that TSRS1 is superior in definition and clarity (Figure 1B). Weak positive NCC patient samples are harder to distinguish on the TS18var1 blot (Figure 1C), and often appear as faint, shadowy bands.

### DISCUSSION

The principal objective of this study was to develop a cysticercosis detection assay that was not reliant on native cyst material, yet equaled the sensitivity and specificity shown by the current seroassay of choice, the native EITB. The development of synthetic and recombinant LLGP antigens has afforded the opportunity to assess the feasibility of supplanting the native EITB as the standard serologic assay for cysticercosis confirmation. The results of the TSRS1 EITB indicate that native antigen is not superior to synthetic antigen for detection of antibodies to NCC in this assay format, showing a sensitivity of 96% and a specificity of 98%, which are comparable to that of LLGP EITB (Table 3). A distinct advantage of the TSRS1 EITB is that only one antigenic band will appear in a positive NCC assay, as opposed to detection of any one of seven antigenic bands for a positive result in the native LLGP EITB.

Although TS18var1 EITB also performed with a high degree of sensitivity (97%) and specificity (100%) against NCC cases of multiple or racemose cysts, it is not a suitable substitute for native antigen in this assay format (Table 3). The TS18var1 EITB only detected 35% of single-cyst cases, whereas TSRS1 and native antigen both detected 85% of those patients. Furthermore, TS18var1 was tested at an ideal gel-loading concentration of 0.001 mg/mL/mm; higher concentrations of TS18var1 were cross-reactive with both NHS and other parasitic disease sera. The ultra-low concentration of this antigen in the EITB is problematic in two ways. First, the positive cases are not as clearly defined, often resulting in very faint bands (Figure 1C). Second, the consistent reproducibility of this assay is not ensured at such a low concentration. Conversely, TSRS1 exhibited no problems of cross-reactivity when tested at higher concentrations, and slight variations of the loading concentration of 0.02 mg/mL/mm did not skew assay results affecting sensitivity and specificity.

The secondary objective of this study was to develop a quantitative confirmatory assay for cysticercosis that may—

### Table 2

Performance of four *Taenia solium* 8-kD antigens in an enzyme-linked immunosorbent assay format when tested against sera from clinically defined neurocysticercosis patients showing various stages of disease progression (n = 206)*

<table>
<thead>
<tr>
<th>Antigen (mg/mL)</th>
<th>% sensitivity</th>
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<tbody>
<tr>
<td></td>
<td>≥ 2 viable and racemose cysts</td>
</tr>
<tr>
<td>TS14 (0.01)</td>
<td>90</td>
</tr>
<tr>
<td>TS18var1 (0.01)</td>
<td>95</td>
</tr>
<tr>
<td>TSRS1 (0.01)</td>
<td>77</td>
</tr>
<tr>
<td>TSRS2var1 (0.01)</td>
<td>86</td>
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</table>

* Serum samples from persons infected with other parasitic diseases from countries where cysticercosis is not endemic (n = 79) and healthy individuals who have not traveled outside the United States (n = 97) were used to determine antigen specificity.
part information regarding patient disease status, as well as lend itself to a high-throughput assay format. Unfortunately, direct coating of the 8-kD synthetic peptides to polystyrene for serodetection of NCC was unsuccessful. The antigens tested were either insensitive to some infected serum, as in the case of TSRS1, or highly cross-reactive with uninfected serum. Although the TS18var1 ELISA had the highest Youden's J index (0.791), its specificity for NCC cases is too low (85%) for accurate interpretation in clinical settings.

Initial ELISA evaluation studies using biotinylated 8-kD antigens sensitized to streptavidin-coated polystyrene yielded promising results. When biotinylated-TS18var1 was further tested in an ELISA against a larger cohort of sera (202 samples), it performed similarly to the direct-coating TS18var1 ELISA in this study (Bueno E, unpublished data). The clinically defined Peruvian patient test serum used here has afforded the most comprehensive analyses of the synthesized 8-kD antigens in the ELISA format to date.

**TABLE 3**

Performance of four *Taenia solium* 8-kD antigens in an enzyme-linked immunoelectrotransfer blot format when tested against sera from clinically defined neurocysticercosis (NCC) patients showing various stages of disease progression (n = 307)*

<table>
<thead>
<tr>
<th>Antigen (mg/mL/mm)</th>
<th>≥ 2 viable and racemose cysts (n = 107)</th>
<th>Single viable cysts (n = 20)</th>
<th>Degenerating cysts (n = 66)</th>
<th>Calcified cysts (n = 114)</th>
<th>% specificity (n = 307)</th>
<th>Youden’s J index</th>
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<tr>
<td>TS14 (0.005)</td>
<td>94</td>
<td>70</td>
<td>55</td>
<td>63</td>
<td>76</td>
<td>0.703</td>
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<tr>
<td>TS18var1 (0.001)</td>
<td>97</td>
<td>35</td>
<td>44</td>
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<td>100</td>
<td>0.972</td>
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<tr>
<td>TSRS1 (0.020)</td>
<td>96</td>
<td>85</td>
<td>61</td>
<td>47</td>
<td>98</td>
<td>0.943</td>
</tr>
<tr>
<td>TSRS2var1 (0.005)</td>
<td>82</td>
<td>40</td>
<td>38</td>
<td>29</td>
<td>98</td>
<td>0.800</td>
</tr>
</tbody>
</table>

* Serum samples from persons infected with other parasitic diseases from countries where cysticercosis is not endemic (n = 162), and healthy individuals who have not traveled outside the United States (n = 146) were used to determine antigen specificity. The Youden's J Index values for all four antigens were similar when calculated using the same NCC patient and control serum samples used in an 8-kD enzyme-linked immunosorbent assay (n = 382), so results of the larger serum cohorts are shown.
Synthetic protein antigens provide a simplified, low-cost alternative to native antigens in diagnostic applications. As cysticercosis eradication efforts progress, native cyst material will become increasingly more difficult to obtain. There are a few examples of attempts to use synthetic peptides to detect cysticercosis. Although all of these published trials used their respective peptide antigens in an ELISA format, only one, the biotinylated 8-kD antigen trial, was a serodetection assay. The others relied on extraction of patient cerebrospinal fluid as the test sample, which is a highly invasive procedure, as well as impractical in most clinical settings where cysticercosis is endemic.

As indicated by this study, assay format has a great bearing on the value of the 8-kD synthetic proteins as cysticercosis detection antigens. TSRS1 performed well in the EITB format in which it was discovered, yet failed to perform with the desired sensitivity and specificity in the ELISA format. The TSRS1 EITB has effectively shown that synthetic antigens can closely match the sensitivity and specificity of native antigen when tested in the appropriate format. Due to the small size of synthetic proteins, and their lack of native structure, both solid- and liquid-phase diagnostic applications merit exploration. Future seroassay development with 8-kD peptides will include direct application of 8-kD synthetic antigens to nitrocellulose, and an ELISA in which the antigen-antibody binding occurs in solution.

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