A recombinant protein and synthetic peptide-based immunoblot
test for diagnosis of neurocysticercosis

John Noh¹, Silvia Rodriguez², Yeuk-Mui Lee¹, Sukwan Handali¹, Armando E.
Gonzalez³,⁴, Robert H. Gilman⁴, Victor C.W. Tsang⁵,
Hector H. Garcia²,⁴,⁶, and Patricia P. Wilkins¹

¹Division of Parasitic Diseases & Malaria, Center for Global Health, Centers for Diseases
Control and Prevention, Atlanta, GA; ²Cysticercosis Unit, Instituto Nacional de Ciencias
Neurologicas, Lima, Peru; ³Bloomberg School of Public Health, Johns Hopkins
University, Baltimore, MD; ⁴School of Veterinary Medicine, Universidad de San Marcos,
Lima, Peru; ⁵Department of Biology, Georgia State University, Atlanta, GA; ⁶Department
of Microbiology and Center for Global Health, Universidad Peruana Cayetano Heredia,
Lima, Peru

Running Title: A recombinant immunoblot for neurocysticercosis

Corresponding author: John Noh, 1600 Clifton Road, Atlanta, GA 30329; phone: 404-
718-4111; fax: 404-718-4191; email: jnoh@cdc.gov
ABSTRACT

One of the best characterized tests for diagnosis of neurocysticercosis (NCC) is the enzyme-linked immunoelectrotransfer blot (EITB) developed at CDC that uses lentil-lectin purified glycoproteins (LLGP) extracted from *T. solium* cysticerci. Although the test is very reliable, purification of the LLGP antigens has been difficult to transfer to other laboratories because of the need for expensive equipment and technical expertise. To develop a simpler assay, we have previously purified and cloned the diagnostic glycoproteins in the LLGP fraction. In this study, we evaluated three representative recombinant or synthetic antigens from the LLGP fraction, individually and in different combinations, using an immunoblot assay (recombinant EITB). Using a panel of 249 confirmed-positive and 401 negative sera the sensitivity of the recombinant EITB was determined to be 99% and the specificity 99% for diagnosis of NCC. We also tested an additional panel of 239 confirmed NCC-positive sera in Lima, Peru and found similar results. Overall our data show that the performance characteristics of the recombinant EITB are comparable to the LLGP-EITB assay. This new recombinant/ synthetic antigen based assay is sustainable and can be easily transferred to laboratories in the U.S. and throughout the world.
Diagnosis of neurocysticercosis (NCC), caused by the larval stages of *Taenia solium*, is reliably established using results from both imaging and serological tests. Guidelines proposed for diagnosis of presumptive NCC (1, 2) define cases as definitive, probable, or possible based on CT or MRI findings, exposure risk, and serological results. These guidelines specifically define the lentil lectin-bound glycoprotein enzyme-linked immunotransfer blot (LLGP-EITB) as the serological reference standard for diagnosis of NCC (3).

The LLGP-EITB is an immunoblot method that detects antibodies to one or more of seven lentil lectin-bound glycoproteins, which are present in the soluble fraction of an extract of *T. solium* cysts. In patients with multiple, enhancing intracranial lesions, the LLGP-EITB is 100% specific and 95% sensitive using serum or CSF, respectively (3-6). The original description and evaluation of the LLGP-EITB was performed using sera from biopsy proven cases of NCC, most with multiple lesions, as detected by skeletal radiographs and reported a sensitivity of 98% and specificity of 100% (3). Continued monitoring of the test performance, compared with clinical findings using newer imaging techniques, such as CT and MRI, revealed that the sensitivity of the assay was lower, between 50 to 80%, in cases with a single lesion or calcified cysts (6-10). The specificity of the LLGP-EITB has been remarkable, essentially 100%, with only rare anecdotal reports of false-positive results (11-13).

Although the LLGP-EITB is an excellent test, purification of the LLGP from cysts collected from naturally infected pigs has been difficult to standardize and the polyacrylamide gel system used for the LLGP-EITB assay has been difficult to establish in other laboratories. Greater availability of simple and reliable diagnostics for NCC is
anticipated through the use of recombinant or synthetic protein antigens. Therefore, we systematically purified and cloned the diagnostic glycoproteins in the LLGP fraction (14-16). We found that the seven diagnostic proteins comprise three distinct antigenic protein families: the gp50, gp24, and 8-kDa families. We chose representative recombinant or synthetic forms from each of the three antigen families and incorporated these into an immunoblot test (recombinant EITB) for the clinical diagnosis of NCC.
MATERIALS AND METHODS

Chemicals and Reagents

All reagents were reagent grade or better and unless otherwise noted were obtained from Mallinckrodt (St. Louis, MO). The horseradish peroxidase labeled goat anti-human IgG secondary antibody was prepared at CDC (17).

Serum Samples

Sera for assay optimization. A serum pool constructed by pooling 5 serum samples from human cases with confirmed cysticercosis was used for optimizing both the assay and as a positive assay control. This pool contained antibodies that reacted with all seven of the diagnostic proteins in the LLGP-EITB. A serum sample from a human case of alveolar echinococcosis (Echinococcus multilocularis) was used as a heterologous infection serum control. A negative serum pool was constructed from 5 serum samples from United States residents with no reported history of international travel.

Serum panels for determinations of diagnostic sensitivity and diagnostic specificity. A total of 249 serum specimens from persons with defined NCC were collected by the Instituto Nacional de Ciencias Neurologicas, Lima, Peru, from patients presenting with clinical symptoms of NCC and provided to CDC for the initial evaluation studies. The definitive diagnosis of NCC was confirmed by CT or MRI brain imaging (1, 2). Serum samples from the NCC cases were sorted into three categories based on the imaging data from each patient: sera from patients with 2 or more viable cysts (n = 107) (these included sera from patients with multiple viable cysts or a racemose cyst), patients with a single, viable cyst (n = 52), and patients with calcified and/or degenerated cysts (n = 90). Cases with a single lesion and that were seronegative were still considered confirmed NCC cases. Cases classified as calcified included patients with single or
multiple calcified cysts, and those categorized as degenerated cysts had one or two
degenerated cysts. All serum samples were collected in compliance with protocols
approved by the ethical review boards of all participating institutions with specific
permission for future use of stored samples.

A total of 401 serum samples were used to assess specificity (Table 1). A panel of
191 serum samples was assembled from healthy residents of the United States (n = 167) or
Egypt (n = 24). The donors from Egypt were tested for the presence of intestinal parasites
by stool examination and all were negative. In addition, a panel of sera collected from
patients with heterologous infections (n = 210) was used. All of these serum samples
were collected in regions where transmission of cysticercosis/taeniasis does not occur,
except the sera from persons with *T. saginata* taeniasis, which were collected in Peru.
Differentiation of *T. solium* and *T. saginata* was made after examining the proglottids or
by PCR (18-20).

Another unique set of serum samples from NCC patients were tested in Lima,
Peru. The 239 defined sera were collected from 119 patients with 2 or more viable cysts
patients, 84 patients with a single viable cyst, and 36 patients with other stages of NCC.

**Recombinant and synthetic *T. solium* protein antigens**

Recombinant rGP50 was expressed in Sf21/Sf9 cells and the hydrophilic
extracellular domain of T24, rT24H, was expressed in the *Tni* cell line, using baculovirus
expression systems, as described previously (15, 16). TsRS1, a 66 amino acid peptide and
a member of the 8-kDa antigen family (21), was chemically synthesized (sTsRS1)
(Anaspec, San Jose, CA).

**SDS-PAGE and EITB**
Proteins were electrophoretically separated by sodium dodecyl sulfate and polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to nitrocellulose (Whatman Protran BA83, Cat. # 10 541 103, 0.2μm pore size) as described previously (22). The blots were cut into 2.5 mm strips and stored in PBS/0.1% NaN₃ at 4°C prior to use. Sera were tested and specific antibodies were detected as described previously (3, 22). Blot strips and the initial experiments to establish the optimal concentration of each recombinant protein/synthetic peptide were prepared using 5-22.5% polyacrylamide gels that were made in our lab at CDC (22). To see if the proteins/peptide could be separated adequately using commercially prepared gels, we used 15% and 4-22% gradient gels from Bio-Rad (Hercules, CA).

**Data Analysis**

Agreement between the LLGP-EITB and recombinant EITB results was determined using Cohen's kappa statistic (23). We used the following scale to interpret kappa: <0, less than chance agreement; 0.01-0.20, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60 moderate agreement; 0.61-0.80, substantial agreement; 0.81-0.99 almost perfect agreement (24).
RESULTS

An initial set of experiments was conducted to determine the optimal concentration of each individual antigen for the recombinant EITB. Each of the protein antigens, rGP50, rT24H, and sTsRS1, was diluted serially and analyzed separately, using SDS-PAGE and immunoblot analysis. The blots were incubated with the pooled cysticercosis serum, the echinococcosis serum, or the pooled normal serum. The optimal concentration of each antigen was determined visually by choosing the concentration that gave the greatest specific reactivity, observed by comparing the reactivity with the cysticercosis serum to reactivity with the echinococcosis serum and the pooled normal serum. The optimal concentrations of the antigens were determined: rGP50, 0.2 ng/mm; rT24H, 2.5 ng/mm; and sTsRS1, 10 ng/mm (data not shown).

The 3 antigens were mixed together at their respective final optimal concentrations and separated using SDS-PAGE. The pooled recombinant proteins, rGP50 and rT24H and the synthetic peptide, sTSRS1, were separated on 5-22.5% polyacrylamide gels made in-house (Figure 1). The three antigens were successfully resolved when the mixture of recombinant proteins/synthetic peptide was separated using commercially prepared polyacrylamide gels (Figure 2).

The sensitivity of the recombinant EITB for diagnosing NCC was assessed at two sites using two different serum batteries: at the CDC in Atlanta, GA, USA and at the Instituto Nacional de Ciencias Neurologicas, Lima, Peru. When the data from both sites are considered, the combination of all three antigens proved to be sensitive than any single antigen alone for detecting cases of NCC with 2 or more viable cysts (Table 2), although the sensitivity was excellent (>95%) for all combinations of antigens except TSRS1 alone, which had a sensitivity for detecting NCC cases of 85% or 75%, depending on the
serum battery examined. When the sensitivity of testing for the individual antigens was evaluated, rT24H performed better than rGP50, than TSRS1 (rT24H.rGP50>TSRS1). Similar patterns of reactivity occurred for sera from NCC patients with a single viable cyst or degenerate or calcified cysts only (Tables 2 and 3).

The specificity of the recombinant EITB for diagnosing NCC was also evaluated using a panel of 401 serum samples (Table 1). Sera for the specificity analysis were collected from healthy individuals (n =191) or patients with heterologous infections (n = 210). The specificity of the recombinant EITB using the recombinant/synthetic antigens, either alone or in combination, was greater than or equal to 98% (Table 2).

We compared reactivity in the recombinant EITB to the currently accepted standard for laboratory diagnosis, the LLGP-EITB (Tables 2 and 4). Concordance between the recombinant EITB and the LLGP-EITB results was almost perfect (kappa = 0.89). Most (10/12) of the non-concordant results occurred because of reactions with the rGP50 antigen only. Two sera from Hymenolepis infections generated false positive results in both the recombinant EITB and the LLGP-EITB and reacted with native 8kDa-protein antigens and TSRS1.

We also compared reactivity with the rT24H antigen to native gp42 and gp24. Similar levels of reactivity were seen using sera from cases with two or more viable cysts with the recombinant and the native proteins (Table 4). The native gp24 and gp42 proteins were more sensitive detecting single cysts infections. The kappa statistic measuring concordance between the recombinant EITB and reactivity to the native gp42 plus gp24 was 0.93, demonstrating an almost perfect degree of concordance.
This study demonstrates that recombinant proteins can replace the native LLGP antigens for diagnosis of NCC with comparable sensitivity and specificity when used in an immunoblot format. The sensitivity of the recombinant EITB was similar to that of the native LLGP-EITB when all 3 antigens were used in combination and when rT24H was used in any combination or alone. The specificity of the new recombinant EITB was also similar to that of the LLGP-EITB. Since its introduction over 30 years ago, the specificity of the LLGP-EITB has remained exquisite, very near 100%, although there have been a few anecdotal reports of false positive results mainly associated with reactivity to the native gp50 protein alone (11-13). In one study, only 2 of 13 patients with antibodies to only native gp50 were associated with a clinical diagnosis of NCC; seven cases had other final diagnoses (12) suggesting that reactivity to gp50 alone should be interpreted with caution. Similarly in this study, the inclusion of rGP50 increases sensitivity in detecting patients with single lesions on CT, however the overall specificity of the assay is reduced when rGP50 is included. In this study cross reactivity was seen in patients with schistosomiasis. This may represent cross-reactivity or may represent undetected exposure to T. solium in these patients. This observation could have implications in regions here the two diseases are endemic.

sTSRS1, instead of one of the other 8 kDa peptides, was specifically selected for use in this study based on data from previous studies. When sTSRS1 was used in an immunoblot format, sTSRS1 detected 96% of NCC cases with 2 or more viable cysts (25). In another study that evaluated the performance of several 8 kDa proteins in a MAPIA assay, sTSRS1 showed at sensitivity of 81% (26). Typically members of the 8 kDa-protein family have been the least sensitive antigens when these peptides were compared
to other LLGP-derived recombinant proteins, with sensitivities ranging from 77% in the MAPIA to 97% using an immunoblot test (25-29). It is unclear why the 8 kDa peptides performed differently in an earlier study using a similar immunoblot test (25). Different serum panels were used in the two comparisons, but no obvious differences in the sera were found on investigation. The addition of the sTsRS1 peptide did not improve the sensitivity or specificity of the recombinant EITB for diagnosis of NCC in clinically ill patients in this study.

Native cyst derived GP39–42 and GP24 are among the more immunodominant antigens in the LLGP fraction, generating antibodies in approximately 95% of cases of NCC with more than one viable cyst (3, 30). Studies have demonstrated that GP39-42 is a dimeric form of GP24 (16), suggesting that a large percentage of NCC cases would be detected using a recombinant form of GP24. In this study, the rT24H antigen alone had a sensitivity (99%) and specificity (100%) for NCC cases with more than one viable cyst, which was comparable to the LLGP-EITB using native proteins. In multiple assay formats, rT24H alone has performed as well or better than the combination of antigens (16, 26, 28). Based on the data from this and other studies using the recombinant LLGP-derived proteins, we propose that rT24H can be used alone and should be an option for laboratories and manufactures to consider, since its use alone may simplify result interpretation and reduce the cost of the assay. Although rT24H can be used alone for most applications, particularly diagnostics, it may be useful to include a representative antigen from all three antigen families in certain situations, such as epidemiological studies to stratify parasite load in pigs (31, 32).

The recombinant EITB has not improved the sensitivity of NCC cases with single cysts. Significant improvement in sensitivity for single cyst cases may not be possible.
Virtually all serological tests for NCC have reduced sensitivity in this scenario and we hypothesize that this is a result of a lower infection burden and thus lower antigenic stimulation, which results in a limited antibody response. The only data that suggests that detection of cases with single cysts can be improved is a preliminary report by Handali, et al which utilized these same recombinant proteins in a multiplex, fluorescent bead-based assay. In that work, the sensitivity for detection of single lesion cases of NCC was 92% (33).

In conclusion, the recombinant EITB using recombinant and synthetic derived proteins based on the native LLGP antigens represents an improvement in laboratory diagnostics for NCC and other assay formats, such as MAPIA and ICT could also be explored for clinical diagnosis (26, 29). Dependence on cyst derived antigens is eliminated thereby lowering the cost and eliminating the lot-to-lot variability associated with native protein antigens. Furthermore, result analysis is simplified, due to less background and the absence of cross-reacting proteins that were sometimes present in native parasite extracts (3). Perhaps equally important, the ability to use commercial manufactured polyacrylamide gels allows implementation in most reference laboratories and bodes well for the commercialization of the method for clinical and epidemiological use or incorporation of the recombinant proteins into other formats.

ACKNOWLEDGEMENTS

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collection, analysis, or interpretation, in writing the manuscript, or in the decision to submit the article for publication.
Table 1. Diagnostic specificity of LLGP-EITB and recombinant EITB

<table>
<thead>
<tr>
<th>Sera classification</th>
<th>N</th>
<th># LLGP-EITB reactive (%)</th>
<th># Recombinant EITB reactive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human sera</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>American non-travelers</td>
<td>167</td>
<td>0</td>
<td>2 (1)*</td>
</tr>
<tr>
<td>Egyptian urbanites</td>
<td>24</td>
<td>0</td>
<td>1 (1)*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-<em>T. solium</em> taeniasis</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>T. saginata* taeniasis</td>
<td>20</td>
<td>4 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Ascariasis</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hydatid echinococcosis</td>
<td>27</td>
<td>8 (30)</td>
<td>0</td>
</tr>
<tr>
<td>Alveolar echinococcosis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Filariasis</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amebiasis</td>
<td>11</td>
<td>0</td>
<td>2 (18)*</td>
</tr>
<tr>
<td>Fascioliasis</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Soil transmitted helminths</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Giardiasis</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heterophyiasis</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hymenolepiasis</td>
<td>10</td>
<td>2 (20)</td>
<td>2 (20)**</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>26</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malaria</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Paragonimiasis</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>54</td>
<td>0</td>
<td>5 (9)*</td>
</tr>
<tr>
<td>Clonorchiasis</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>**Total</td>
<td>401</td>
<td>14(3)</td>
<td>12 (3)</td>
</tr>
</tbody>
</table>

*   = positive against rGP50 only
** = positive against TsRS1 only
Table 2. Sensitivity and specificity of the recombinant EITB test performed at two sites, at the CDC, Atlanta, USA and the Instituto Nacional de Ciencias Neurologicas, Lima, Peru.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Testing Site</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2+ cysts</td>
<td>single cyst</td>
<td></td>
</tr>
<tr>
<td>LLGP Lima</td>
<td>100</td>
<td>79</td>
<td>ND</td>
</tr>
<tr>
<td>Atlanta</td>
<td>96</td>
<td>52</td>
<td>ND</td>
</tr>
<tr>
<td>rGP50 + rT24H + TsRS1 Lima</td>
<td>97</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td>Atlanta</td>
<td>99</td>
<td>56</td>
<td>98</td>
</tr>
<tr>
<td>rGP50 + rT24H Lima</td>
<td>96</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td>Atlanta</td>
<td>99</td>
<td>54</td>
<td>99</td>
</tr>
<tr>
<td>rGP50 + TsRS1 Lima</td>
<td>92</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>Atlanta</td>
<td>97</td>
<td>50</td>
<td>98</td>
</tr>
<tr>
<td>rT24H + TsRS1 Lima</td>
<td>96</td>
<td>52</td>
<td>98</td>
</tr>
<tr>
<td>Atlanta</td>
<td>99</td>
<td>48</td>
<td>99</td>
</tr>
<tr>
<td>rGP50 Lima</td>
<td>89</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>Atlanta</td>
<td>96</td>
<td>46</td>
<td>99</td>
</tr>
<tr>
<td>rT24H Lima</td>
<td>96</td>
<td>52</td>
<td>98</td>
</tr>
<tr>
<td>Atlanta</td>
<td>99</td>
<td>44</td>
<td>100</td>
</tr>
<tr>
<td>TsRS1 Lima</td>
<td>85</td>
<td>39</td>
<td>100</td>
</tr>
<tr>
<td>Atlanta</td>
<td>75</td>
<td>21</td>
<td>99</td>
</tr>
</tbody>
</table>

ND, not determined
Table 3. Sensitivity of the recombinant EITB test against samples with degenerated or calcified cyst(s) performed at CDC, Atlanta, USA.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Degenerated cysts only</th>
<th>Calcified cysts only</th>
</tr>
</thead>
<tbody>
<tr>
<td>rGP50 + rT24H + TsRS1</td>
<td>83</td>
<td>78</td>
</tr>
<tr>
<td>rGP50 + rT24H</td>
<td>74</td>
<td>78</td>
</tr>
<tr>
<td>rGP50 + TsRS1</td>
<td>74</td>
<td>64</td>
</tr>
<tr>
<td>rT24H + TsRS1</td>
<td>70</td>
<td>76</td>
</tr>
<tr>
<td>rGP50</td>
<td>65</td>
<td>64</td>
</tr>
<tr>
<td>rT24H</td>
<td>61</td>
<td>73</td>
</tr>
<tr>
<td>TsRS1</td>
<td>35</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 4. Comparison of test sensitivity using the LLGP-EITB versus the recombinant EITB, performed at the CDC, Atlanta, USA and the Instituto Nacional de Ciencias Neurologicas, Lima, Peru.

<table>
<thead>
<tr>
<th>Assay, Reactivity</th>
<th>Sensitivity detecting cases with 2 or more viable cysts (%)</th>
<th>Sensitivity detecting cases with Single viable cyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Atlanta</td>
<td>Lima</td>
</tr>
<tr>
<td>LLGP-EITB, any band</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>Recombinant EITB, any band</td>
<td>99</td>
<td>98</td>
</tr>
<tr>
<td>LLGP-EITB, native gp42 + native gp24</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>Recombinant EITB, rT24H only</td>
<td>99</td>
<td>96</td>
</tr>
</tbody>
</table>
REFERENCES


**FIGURE LEGENDS**

Figure 1. Recombinant protein immunoblot analysis. rGP50, rT24H, TsRS1 separated using SDS-PAGE with the CDC polyacrylamide gel system. Strips were incubated with sera from patients with NCC, (B) heterologous other parasitic infections, or (C) healthy Americans.

Figure 2. Resolution of the recombinant and synthetics antigens using commercially available polyacrylamide gels. rGP50, rT24H, TsRS1 were applied to 2 commercially available polyacrylamide gels then transferred to nitrocellulose. The proteins were visualized using a pool of cysticercosis sera and immunoblot analysis. (1) 15% polyacrylamide gel, (2) 4-20% polyacrylamide gel.