

APPLICATION OF SYNTHETIC 8-KD AND RECOMBINANT GP50 ANTIGENS IN THE DIAGNOSIS OF NEUROCYSTICERCOSIS BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract. The gold standard serodiagnostic assay for cysticercosis and neurocysticercosis, diseases caused by the metacestode of *Taenia solium*, uses lentil lectin-purified glycoprotein (LLGP) in a Western blot assay. We tested two antigens derived from LLGP, synthetic TS18var1 (sTS18var1) and recombinant GP50 antigen (rGP50), in an enzyme-linked immunosorbent assay (ELISA) using serum and cerebrospinal fluid (CSF) samples. The sensitivity for serum and CSF was 94.7% and 100% for rGP50 and 90.4% and 90.2% for sTS18var1, respectively. The specificity for serum and CSF samples was 93.8% and 100% for rGP50 and 90.3% and 98.0% for sTS18var1, respectively. The use of these antigens individually or combined as a diagnostic antigen cocktail eliminates the need for purification of antigens from parasite material and offers the advantage of using a simple and quantitative ELISA format.

INTRODUCTION

Neurocysticercosis is one of the most common infections of the nervous system in humans. It is caused by the presence of the metacestode, or larval stage, of the tapeworm *Taenia solium* in tissues of the central nervous system.^{1–3} The disease affects millions of people worldwide and is a major public health problem for most of the developing world.^{4,5} A recent estimate of the prevalence of neurocysticercosis in Latin America indicated that 400,000 people have symptomatic disease and many more are infected, but not yet symptomatic.⁶ In addition, neurocysticercosis has emerged in developed countries that receive immigrants from endemic areas.^{7–9}

The symptoms of neurocysticercosis are nonspecific and are of little diagnostic value. Symptoms include seizure, headache, intracranial hypertension, and hydrocephalus.^{10,11} For diagnosis, image studies such as computed tomography (CT) and magnetic resonance (MRI) are recommended.¹² However, the high cost of these techniques limits their accessibility to a few patients, excluding most of the population in disease-endemic rural areas.¹³ Immunologic assays for the detection of cysticercosis-specific antibodies are a valuable tool and may be used either alone or in conjunction with brain imaging.^{12,14} In addition, immunologic assays are essential for determining the prevalence of *T. solium* infection, identifying areas of disease transmission, and monitoring of sentinel pigs for cysticercosis.^{15–18} Since 1995, the gold standard antibody-based diagnostic assay has been the Centers for Disease Control and Prevention (CDC) Western blot, or enzyme-linked immunoelectrotransfer blot (EITB), using lentil lectin purified glycoproteins (LLGP) as antigen and serum as the biologic sample.^{12,19,20} The assay has a specificity of 100% and a sensitivity of 98% for detection of two or more cysts.¹⁹ However, the sensitivity for detecting single cysts is lower.^{21–24} Taking into account that the LLGP antigen is a mixture of proteins that has not been successfully used in any format other than Western blot, and that the source of antigen is the cysticerci from naturally infected pigs, which will become more difficult to obtain as eradication efforts are successful, the LLGP antigens were previously targeted for cloning, sequencing, and expression or synthesis.^{25–28}

Recently, two *T. solium* proteins were produced and char-

acterized, the 8-kD antigens^{26–28} and the GP50 antigen.²⁵ The 8-kD antigens are a family of diagnostic proteins seen at 14, 18, and 21 kD on the LLGP Western blot and are also found in the 24- and 39–42-kD bands.²⁶ One of the 18 8-kD proteins, TS18 variant 1 (sTS18var1, AF098073), was chemically synthesized. It had a sensitivity of 97% and specificity of 100% in a previous study using an enzyme-linked immunosorbent assay (ELISA) format and was considered one of the best choices of the 8-kD family of proteins for antigen for use in a diagnostic assay.²⁶ *Taenia solium* recombinant GP50 antigen (rGP50, AY212945), in a previous, small-scale study using a Western blot assay, had a sensitivity of 90% and a specificity of 100%.²⁵ In the present study, we test the use of sTS18var1 and rGP50 in an ELISA for the diagnosis of neurocysticercosis, using both serum and cerebrospinal fluid (CSF) as the diagnostic sample, and compare the sensitivity of the synthetic and recombinant antigens to that of the corresponding native proteins.

MATERIAL AND METHODS

Sample collection. A total of 64 paired serum and CSF samples from patients with neurocysticercosis from Brazil were studied. The patients were selected according to the General Neurocysticercosis Investigation Protocol of the Hospital of the Faculty of Medicine and Faculty of Medicine of Ribeirão Preto, University of São Paulo (reviewed and approved by Ethics Committee for the Analysis of Research Projects of the Clinical Director's Office of the Hospital, approval no. 072/97), by the Ethics Committee for the Analysis of Research Projects of the Faculty of Pharmacy, University of São Paulo (approval number 188/2003) and by CDC Institutional Review Board review protocol 3987).

All patients had a definitive diagnosis of neurocysticercosis, according to the diagnostic criteria previously proposed: two major criteria (lesions highly suggestive of neurocysticercosis on imaging studies and a positive serum EITB for the detection of anti-cysticercal antibodies), one minor criteria (positive CSF ELISA result for the detection of anti-cysticercal antibodies²⁹), and one epidemiologic criteria (individuals coming from or living in an area where cysticercosis is endemic).¹² On the basis of CT or MRI, 59 (92.2%) patients

showed the presence of inflammatory signs (active form) and 5 (7.8%) patients lacked evidence of an inflammatory process (inactive form). Also according to the imaging studies, 16 (25.0%) patients had one cyst, 18 (28.1%) patients presented with 2–4 cysts, and 30 (46.9%) patients showed ≥ 5 cysts. The average age of the patients was 38.8 years, ranging from 17 to 71 years, 41 (64.1%) were male, and 23 (35.9%) were female.

The control group included 110 serum samples from healthy individuals and 50 CSF samples from patients with non-cysticercosis neurologic disorders. Both sets of samples were from Brazil and showed no reactivity with LLGP on Western blot. The other parasitic infection group was formed by 35 serum samples from patients with parasitologically confirmed infections, other than cysticercosis or taeniasis by *T. solium*, from the CDC serum collection (2 *Ascaris lumbricoides*, 5 *Echinococcus granulosus*, 1 *E. multilocularis*, 3 *Fasciola hepatica*, 1 *Leishmania donovani*, 6 *Schistosoma haematobium*, 7 *S. mansoni*, 8 *Taenia saginata*, and 2 *Trichinella* spp.). All of these samples were from cases originating in geographic regions that are not endemic for cysticercosis.

Antigens. The 8-kD antigen sTS18var1 was chosen over another 8-kD antigen (sTSRS1) for use as the antigen in this study based on preliminary studies that identified sTS18var1 as a better candidate for a diagnostic antigen because of higher ELISA optical density values with positive serum samples. The mature protein sequence of TS18var1 (AF098073), a total of 66 amino acids, was chemically synthesized by AnaSpec Inc. (San Jose, CA).²⁶ The protein was solubilized in 0.05 M HEPES, 0.1 M NaCl, 2 mM dithiothreitol, pH 7.0, and stored in 43.5% glycerol at -80°C . The mature protein sequence of rGP50, amino acids 17 through 276, was expressed in High FiveTM cells (Invitrogen, San Diego, CA) using a baculovirus expression system and the recombinant protein was purified from culture supernatant by $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by ion exchange chromatography, as previously described.²⁵ The protein concentrations of sTS18var1 and rGP50 were determined by absorbance at 280 nm using the extinction coefficients calculated from the protein sequence.

Antibody detection by ELISA. The Falcon assay screening test lids and plates are no longer available, but we do call our quantitative ELISA FAST-ELISA. Serum and CSF antibody titers were assessed by FAST-ELISA as described previously.^{26,30} Either rGP50 or sTS18var1 was used to coat the polystyrene sticks of the Nunc Immuno TSP lid (Nunc cat. No. 445497; Nalge Nunc International, Rochester, NY) using the corresponding 96 microwells plate (Nunc cat. No. 269620). rGP50 was coated at a saturating concentration of 1 $\mu\text{g}/\text{mL}$ and sTS18var1 was coated at a saturating concentration of 10 $\mu\text{g}/\text{mL}$. Both antigens were diluted in phosphate-buffered saline (PBS), pH 7.2, (0.01 M phosphate, 0.15 M NaCl) and coated for two hours or longer. The wash buffer and diluent buffer for the conjugate were PBS plus 0.3% Tween 20 (Calbiochem-Novabiochem Corp., La Jolla, CA). Serum and CSF samples were tested in triplicate in the same buffer with 5% nonfat dry milk. The CSF samples were tested at 1:10 for both rGP50 and sTS18var1, while serum samples were tested at 1:33 for rGP50 and 1:100 for sTS18var1. Bound antibodies were detected with peroxidase-labeled goat anti-human IgG,³¹ followed by the substrate SureBlue (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). All reagents except the sera and CSF were present in excess and

all reagents were used at a volume of 150 μL per well. Sample, conjugate, and substrate incubations were five minutes. Between each incubation, the lid with the sticks was held vertically and sprayed using a pressurized garden sprayer containing PBS/Tween 20. The absorbance at 650 nm (A_{650}) was read with a ThermoMax microplate reader (Molecular Devices Corp., Sunnyvale, CA).

A human IgG standard curve was included on each plate. Purified human IgG was quantified using the standard Bradford protein assay and standards ranging from 0 to 5 μg of IgG/mL (0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 2.5, and 5 $\mu\text{g}/\text{mL}$) were constructed by diluting the IgG in PBS with 0.01% bovine serum albumin factor V and 0.1% NaN_3 . The standards were stored at 4°C and used in the ELISA as antigen. During the antibody detection step, the coated sticks were incubated in PBS/Tween 20 with 5% nonfat dry milk. Conjugate and substrate incubations were those used above. Because $\mu\text{g}/\text{mL}$ of IgG in the coating buffer does not translate to $\mu\text{g}/\text{mL}$ of specific IgG in the serum and CSF samples, the values of the standard curve are considered to be arbitrary units of IgG and are referred to as units. In addition to the standard curve, each plate contained a normal human sera pool, a low positive reference standard, and a medium positive reference standard used as controls and tested as samples. These controls were used to validate the results of each plate. If the mean result of the triplicate of these controls on one plate was greater than the mean of all triplicates ± 2 SD, then the data for that plate were discarded and the plate was repeated. The sample dilution used in the assays was taken into account by multiplying each sample result by the respective dilution factor: 33 for serum samples when tested with rGP50, 100 for serum samples when tested with sTS18var1, and 10 for CSF samples when tested with rGP50 and sTS18var1. Determination of the cut-off value discriminating a positive result from a negative one was based on the analysis of the diagnostic efficiency according to the J index.³²

RESULTS

The sensitivity and specificity of recombinant GP50 and synthetic TS18var1 in an ELISA was compared with the sensitivity and specificity of the corresponding native proteins in LLGP as tested on Western blot. Only serum and CSF samples reactive with the corresponding native protein were used in this analysis. rGP50, when tested as an antigen in the ELISA with serum samples, has a sensitivity of 94.7% (54 of 57) and a specificity of 93.8% (136 of 145) (Figure 1A). For sTS18var1, the sensitivity of the ELISA with serum samples is 90.4% (47 of 52) and the specificity is 90.3% (131 of 145) (Figure 1B). The false-positive results, from the other parasitic infection group of serum samples, did not cluster with any particular parasitic infection. rGP50, when tested as an antigen in the ELISA with CSF samples, has a sensitivity of 100% (53 of 53) and a specificity of 100% (50 of 50) (Figure 2A). For sTS18var1, the sensitivity of the ELISA with CSF samples is 90.2% (36 of 41) and the specificity is 98.0% (49 of 50) (Figure 2B).

All 64 patients with a definitive diagnosis of neurocysticercosis had both serum and CSF samples available for evaluation. The reactivity of all 64 serum samples with sTS18var1 antigen in the ELISA was compared with the reactivity of all 64 CSF samples (Figure 3A). No difference in the level of

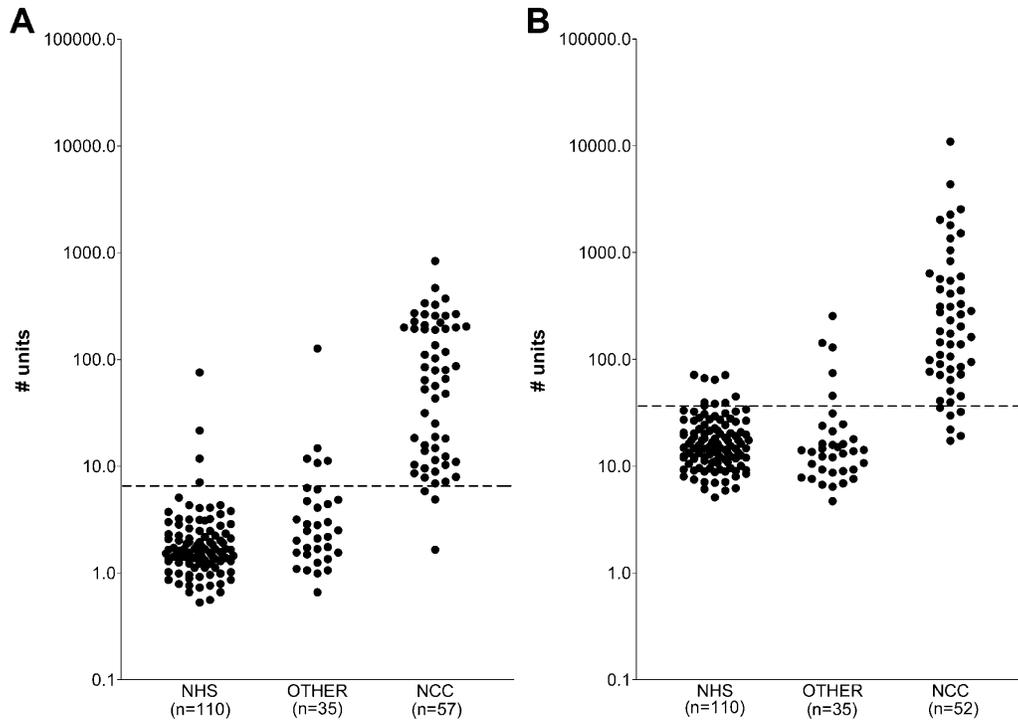


FIGURE 1. Quantitation of serum antibody to **A**, recombinant GP50 and **B**, synthetic TS18var1 antigens by enzyme-linked immunosorbent assay. Serum samples are grouped on the x-axis according to their classification as normal human sera (NHS), other parasite infection sera (OTHER), or neurocysticercosis patient sera (NCC). The cut-off value, determined by selecting the maximum J index, is shown by the dashed horizontal line. The y-axis, in log scale, shows the number of units of IgG according to a standard curve.

reactivity of serum and CSF samples from each patient was seen. The coefficient of determination (r^2) was 0.92. However, when the reactivity of serum and CSF samples from patients was compared using rGP50 as the antigen in the ELISA, several serum samples showed high levels of reactivity, but the corresponding CSF samples showed low levels of reactivity ($r^2 = 0.55$) (Figure 3B). The concordance between positive and negative results of paired serum and CSF samples, with both samples showing the same result, is 89.1% (57 of 64) for rGP50 and 71.9% (46 of 64) for sTS18var1. The sensitivity when using paired serum and CSF samples, with a patient considered positive if the serum and/or CSF test result positive, is 92.2% (59 of 64) for rGP50 and 89.1% (57 of 64) for sTS18var1.

The results from both ELISAs, one using rGP50 and the other using sTS18var1, were combined to evaluate the ability to diagnose neurocysticercosis using these two antigens (Figure 4). All serum and CSF samples from the 64 patients with a definitive diagnosis of neurocysticercosis were included. A sample reactive with rGP50 and/or sTS18var1 was considered positive. The combined sensitivity was 93.8% (60 of 64) for serum samples and 85.9% (55 of 64) for CSF samples. The specificity of the assay was 84.1% (122 of 145) for serum samples and 98.0% (49 of 50) for CSF samples. If only active neurocysticercosis cases are considered, the combined sensitivity for serum samples is 96.6% (57 of 59), while the sensitivity for CSF is 88.1% (52 of 59). The combined sensitivity for single cysts is 81.3% (13 of 16) in serum samples and 75.0% (12 of 16) in CSF samples. If paired serum and CSF samples are tested with both rGP50 and sTS18var1 antigens, the sensitivity increases to 96.9% (62 of 64). For active neu-

rocysticercosis cases, testing with paired samples increases the sensitivity to 98.3% (58 of 59). For single cyst cases, the sensitivity increases to 87.5% (14 of 16).

DISCUSSION

Evaluation of rGP50 in an ELISA format, using samples reactive with the GP50 component of LLGP in a Western blot, showed a sensitivity of 94.7% when serum samples (Figure 1A) were tested and 100% when CSF samples (Figure 2A) were tested. When sTS18var1 was evaluated in the ELISA format, using samples reactive with the 8-kD components of LLGP in a Western blot, the sensitivity was 90.4% for serum (Figure 1B) and 90.2% for CSF (Figure 2B). A preliminary examination using serum samples and rGP50 in a Western blot assay reported a slightly lower sensitivity for rGP50 of 90%,²⁵ while another study using serum samples and sTS18var1 in an ELISA reported a higher sensitivity for sTS18var1 of 97%.²⁶

If one considers that the serum and CSF samples evaluated in each assay were all reactive with the corresponding native protein, the sensitivity of the recombinant and synthetic proteins, as tested in the ELISA, is less than the sensitivity of the native proteins in Western blot. For rGP50, there is a 5% decrease in sensitivity; for sTS18var1, the decrease is 10%. The decrease in sensitivity may be due to the slightly lower sensitivity of ELISA compared with Western blot.²¹ However, it is also possible that differences between the native proteins and the recombinant and synthetic proteins may be contributing to the decrease in sensitivity. Native GP50 and

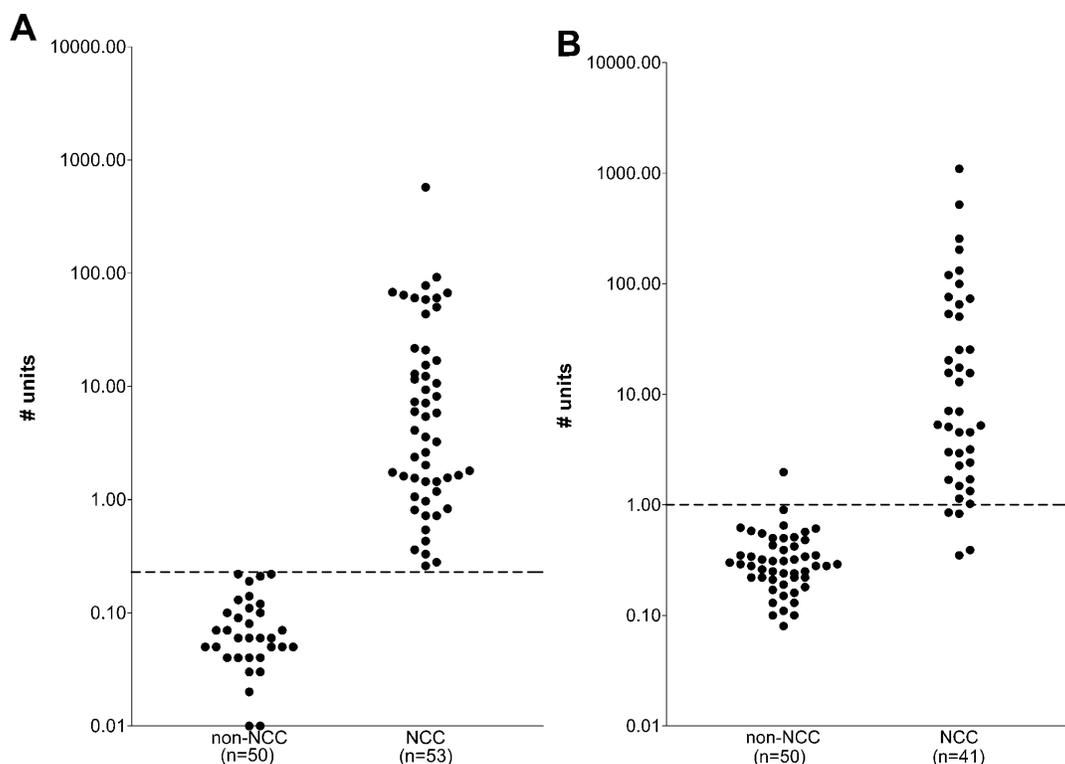


FIGURE 2. Quantitation of cerebrospinal fluid (CSF) antibody to **A**, recombinant GP50 and **B**, synthetic TS18var1 antigens by enzyme-linked immunosorbent assay. The CSF samples are grouped on the x-axis according to their classification as patients with non-cysticercosis neurologic disorders (non-NCC) or neurocysticercosis patients (NCC). The cut-off value, determined by selecting the maximum J index, is shown by the dashed horizontal line. The y-axis, in log scale, shows the number of units of IgG according to a standard curve.

TS18var1 are glycosylated proteins.^{19,26} rGP50 is also glycosylated, but it is glycosylation as defined by the insect cells that the protein was expressed in. Glycosylation in insect cells is different from glycosylation in mammalian cells and is likely to be different from the glycosylation that occurs in *T. solium*.^{33,34} For native GP50, a glycosyl-phosphatidylinositol-anchored protein, the anchor may play a role in its antigenicity.^{35–39} The recombinant protein was expressed without the anchor attachment sequence. For sTS18var1, which is chemically synthesized, it may be the lack of glycosylation, or another post-translational modification, that is impacting upon its sensitivity compared with the native 8-kD proteins. In addition, there are at least 18 8-kD proteins and the native 8-kD proteins form disulfide-bonded multimers whose individual compositions are unknown.²⁶ The lack of comparable multimers in the sTS18var1 may be negatively impacting its sensitivity.

Both antigens were evaluated for specificity. For rGP50, the specificity for serum and CSF samples was 93.8% and 100%, respectively (Figures 1A and 2A). For sTS18var1, the specificity was 90.3% for serum (Figure 1B) and 98.0% for CSF (Figure 2B). The specificity reported for LLGP, the antigen mixture containing native GP50, the native 8-kD proteins, and a third diagnostic protein, T24, with serum samples is 100%.¹⁹ However, there have been a few reports of reactivity with the native GP50 band only in cases that did not turn out to be due to neurocysticercosis.^{24,40} Like the decrease in sensitivity, the decrease in specificity may be related to the non-native or the lack of post-translational modifications. Another factor that may be playing a role are the dif-

ferences between how an antigen is bound to a polystyrene matrix and presented for antibody recognition compared with the binding and presentation of a sodium dodecyl sulfate-treated antigen on nitrocellulose membrane by Western blotting. The issues of lower sensitivity and specificity with the recombinant and synthetic proteins are being addressed.

The gold standard for serodiagnosis of cysticercosis, the Western blot using LLGP as antigen, is considered positive if any one of the diagnostic antigens is recognized by patient's serum. For this reason, an assay relying on a single antigen may lack the required sensitivity. Combining the results from assaying all 64 neurocysticercosis serum samples with rGP50 and sTS18var1, regardless of which band is recognized on Western blot, and considering a sample positive if it reacts with one or both antigens results in a diagnostic sensitivity of 93.8% and a specificity of 84.1%. Using both assays, the sensitivity for detecting a case with a single cyst is 81.2%. Combining the results increases the sensitivity for detecting neurocysticercosis cases, but also increases the risk of false-positive reactions because of the loss of specificity. The decrease in specificity is due to the lack of overlap between sera that are falsely positive in one ELISA with sera that are falsely positive in the other ELISA.

An accurate and rapid assay for diagnosis of neurocysticercosis is essential to implement cysticercosis control programs. Although the gold standard antibody-based diagnostic assay has been the CDC Western blot, an ELISA format is preferred because of ease of use and lower cost. We have evaluated two of the three diagnostic antigens in LLGP in an ELISA. When both recombinant GP50 and synthetic

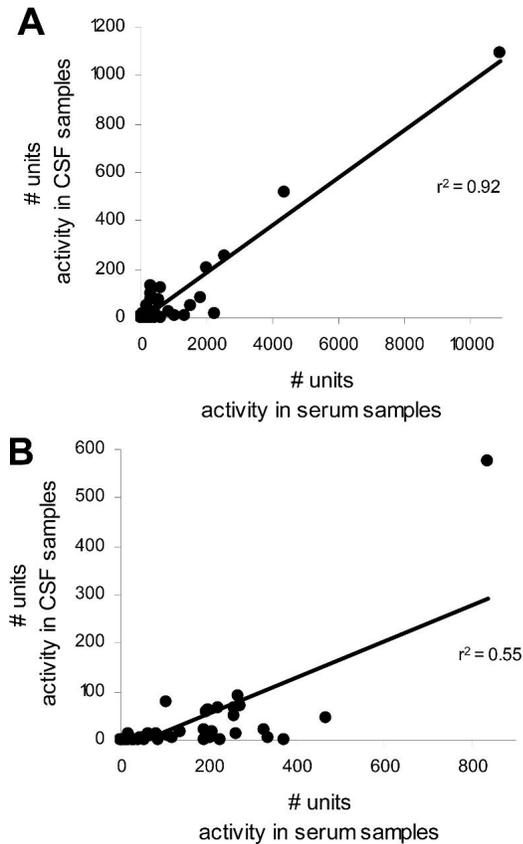


FIGURE 3. Comparison of the antibody reactivities of the samples from 64 patients with a definitive diagnosis of neurocysticercosis. **A**, Antibody reactivity with synthetic TS18var1 antigen in serum samples compared with the antibody reactivity in cerebrospinal fluid (CSF) samples. **B**, Antibody reactivity with recombinant GP50 antigen in serum samples compared with the antibody reactivity in CSF samples. The axes show the number of units of IgG according to a standard curve. The r^2 value for each line is shown.

TS18var1 are used to evaluate a serum sample, the assay sensitivity is 94% and the specificity is 84%. In certain situations, it may be desirable to confirm an ELISA-positive result with the LLGP Western blot assay, and in certain individual cases it may be desirable to test paired serum and CSF samples. The use of these two cloned antigens eliminates the dependence on a source of parasite raw material and allows for antibody testing using the simple and quantitative ELISA format. The addition of the third diagnostic component of LLGP, T24, should improve the sensitivity of the ELISA for the detection of neurocysticercosis.

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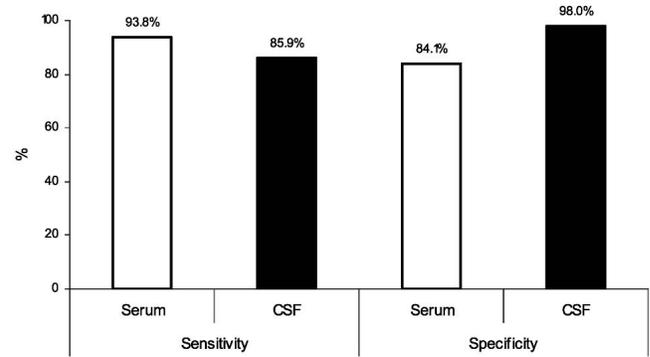


FIGURE 4. Sensitivity and specificity for diagnosing neurocysticercosis using both recombinant GP50 (rGP50) and synthetic TS18var1 (sTS18var1) antigens. Serum samples (**white bars**): 64 patients with a definitive diagnosis of neurocysticercosis, 110 healthy individuals, and 35 individuals with other parasitic diseases. Cerebrospinal fluid (CSF) samples (**black bars**): 64 patients with a definitive diagnosis neurocysticercosis and 50 individuals with other neurologic disorders, non-cysticercosis. A sample reactive with rGP50 and/or sTS18var1 was considered positive.

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