

SERODIAGNOSTIC ASSAYS FOR *TAENIA SOLIUM* CYSTICERCOSIS

by

CHRISTINA MARIE SCHEEL

(Under the Direction of Victor C. W. Tsang)

ABSTRACT

Programs to control transmission of *Taenia solium* cysticercosis will require a simple, yet efficacious serodiagnostic assay for large-scale surveillance of endemic populations to identify reservoirs of disease. To implement large-scale testing, two criteria must be met; the assay must be produced from an unlimited source of diagnostic material, and must have a rapid, low technology format. To meet these criteria, four synthetic *T. solium* antigens, TS14, TS18var1, TSRS1 and TSRS2var1, were applied to an enzyme-linked immunosorbent assay (ELISA) to determine their diagnostic potential. To compare the diagnostic efficacy of these antigens on a universal scale that was free of immunological bias, we constructed a standard reference curve using normal human immunoglobulin G (hIgG). The hIgG standard curve was utilized in each assay plate to control for interassay variations. Upon testing the synthetic antigens in ELISA against defined infection serum cohorts, we found that TS18var1 was the most sensitive for detecting cysticercal antibody, and TSRS1 the most specific, yet none of the antigens performed with high efficacy. Subsequently, each antigen was tested against the serum cohorts in the immunoblot format in which they were originally defined. Two of the antigen candidates,

TSRS1 and TS18var1, proved to be of high sensitivity (96% and 98%, respectively) and high specificity (98% and 100%, respectively) when applied to this format.

Although synthetic antigens provide an unlimited source of diagnostic material, the immunoblot is not a rapid, low-tech assay format. We partnered with a commercial diagnostic laboratory, Immunitics Inc., to apply synthetic TS18var1 and TSRS1, as well as a recombinant antigen, rGP50 to a patented assay format, the QuickELISA™. All three antigens proved efficacious in this format. The quantitative aspect of the QuickELISA™ assays prompted us to explore the possibility of correlating immune response with disease status. We found that TS18var1 and rGP50 showed significant correlations ($P = 0.01$, $P = 0.02$, respectively) between improved disease and decreased seroreactivity in longitudinal serum samples of clinically defined neurocysticercosis patients. The cysticercosis QuickELISA™ assays are suitable for large-scale disease surveillance and may also provide clinicians with a practical tool for patient management.

INDEX WORDS: Cysticercosis Diagnosis *Taenia solium* Immunoassay Neurocysticercosis ELISA Tapeworm Cestode

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To my family
and
in memory of
Tena Marie Kiehne

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

TAENIA SOLIUM

The cestode tapeworm, *Taenia solium*, requires both human and porcine hosts to complete its life cycle. Humans are the definitive hosts; they harbor the adult tapeworm after ingestion of undercooked pork infected with larval cysts, resulting in the disease taeniasis. The porcine intermediate host becomes infected with *Taenia* larva by consuming human feces containing gravid proglottids shed by adult worms. Humans may also act as intermediate hosts upon accidental ingestion of *T. solium* eggs, resulting in cysticercosis.

The scolex of *T. solium* evaginates in the small intestine upon exposure to host bile, and bears a rostellum armed with two rows of 22 to 32 hooks (Wardle and others 1974) that aid in attachment to the intestinal mucosa. The strobila of the adult tapeworm can reach lengths up to four meters (Garcia, Gonzalez and others 2003) and is comprised of a linear series of 700 to 1000 segments (Pawlowski 2002), or proglottids, that house both male and female reproductive organs. As the parasite grows, new proglottids are formed behind the neck, and mature as they are moved toward the posterior end. Genitalia in the proglottids at the posterior end of the tapeworm have completed copulation and are gravid, containing up to 60,000 eggs (Flisser 1994). The terminal gravid proglottids detach, and are shed sporadically from the host in fecal material.

Although many eggs are discharged from the proglottid through an anterior pore, some remain within the uteri and can survive for many months protected by the surrounding tegument and feces (Gemmell and Lawson 1982, 1989; Pawlowski 2002). A single tapeworm can shed as many as 300,000 eggs per day (Lawson and Gemmell 1983). Each egg encloses an invasive hexacanth, or six-hooked, onchosphere that is protected by a globular embryophore that is 31 to 43 μm in diameter (Laclette and others 1982). The embryophore protects the onchosphere from environmental conditions, but disintegrates upon contact with gastric fluids (Lawson and Gemmell 1983). After onchospheres are liberated, they cross the intestinal wall through mesenteric venules and migrate to various organs and tissues, such as the liver, lungs, muscles and brain (Yoshino 1933b; Pawlowski 2002).

Once the onchospheres have migrated, they encyst within the host tissue and develop into the metacestode stage of the parasite (Yoshino 1933a, 1933c). Onchosphere invasion into host tissue causes the formation of a cavity that is lined with epithelioid cells originating from small blood vessels (Pawlowski 2002). The onchosphere then differentiates from a solid larva into a vesicular bladderworm surrounded by a layered membrane (Laclette and others 1982). The scolex forms within the bladder on a germinal inner membrane layer (Schmidt and others 1996), while the cuticular layer provides protection and acts as a trophoblast, absorbing nutrients and excreting waste by diffusion (Ramirez-Bon and others 1982). These metamorphic processes occur very quickly; the metacestode reaches its full size (8 to 10 mm) (Carpio 2002) within 70 days (Pawlowski 2002). The metacestodes are generally tolerated by the host for several years, but eventually begin to degenerate (Dixon and Lipscomb 1961), at which point they come under attack by the host immune system, causing inflammation. Because the lifespan of domestic pigs is ordinarily less than one year, metacestodes do not reach the degeneration stage in these

animals (Garcia, Gonzalez and others 2003). When humans inadvertently become the intermediate host for *T. solium*, severe pathology may occur in the tissues of the central nervous system (CNS).

PATHOLOGY

Cysticercosis occurs when humans accidentally ingest *T. solium* eggs deposited by tapeworm carriers living or working in their community. Between 5 and 40% (Schantz and others 1998) of persons with taeniasis will self-infect via the fecal oral route. Human cysticercosis infections often involve the subcutaneous tissues, brain, and less often, the eye. Subcutaneous infections are fairly innocuous, having few pathological repercussions (Garcia and Del Brutto 2000; Del Brutto and others 2001; Garcia, Gonzalez and others 2003). Ocular cysticercosis may result in blindness, but the most severe form of disease occurs with infections of the CNS and brain tissue, resulting in neurocysticercosis (NCC). Severity of NCC is dependent on both the locality and burden of infection (Nash and Neva 1984; Evans and others 1997; Garcia and Del Brutto 2000; Garcia and others 2002). Metacestodes that inhabit the parenchyma of the brain are limited to sizes no larger than 10 mm due to pressure from the surrounding tissue, whereas those invading the ventricular and subarachnoid spaces may grow as large as 5 cm (Garcia and Del Brutto 2000). Subarachnoid cysticerci that locate to the basal cerebrospinal fluid cisterns often undergo membrane “budding”, resembling clustered grapes (Bickerstaff and others 1952; Rabiela and others 1989). These racemose cysts may reach sizes larger than 10 cm (Garcia and Del Brutto 2000), resulting in severe disease complications, even death.

In general, most of the pathology associated with NCC occurs when the metacestodes begin the degeneration process, resulting in a loss of protection from immunological attack (Dixon and Lipscomb 1961; White and Shandera 1992). The progression of cysticerci devolution may take several years, and has been described as occurring in four distinct stages, viable (vesicular), colloidal (inflamed), granular nodular and calcified cysts (Escobar 1983). Both viable and colloidal, or inflamed cysts are considered to be biologically active, whereas cysts that become granular or calcify are biologically inactive (Carpio and others 1994; Garcia, Gilman, Horton and others 1997; Garcia and others 2002; Garcia and others 2004). It is often the case that infected patients have cysticerci exhibiting more than one stage of degeneration at a given time (Escobar 1983). Although many cases of NCC are asymptomatic throughout, symptoms that do occur may remain long after cysts have calcified. Symptoms of NCC range broadly in severity, but epilepsy is the most common presentation of the disease (Garcia and Del Brutto 2000). Other clinical manifestations include headaches, hydroencephaly and brain atrophy.

DIAGNOSIS AND TREATMENT

Primary diagnosis of NCC is usually performed by either computed tomography (CT) or magnetic resonance imaging (MRI) in cases where symptomatic individuals seek treatment. The various devolutionary stages of cyst lifespan (Escobar 1983) are clearly distinguishable by neuroimaging (Kramer and others 1989). Viable cysts show little evidence of inflammation (Garcia and Del Brutto 2000; Garcia and others 2002; Pawlowski 2002) and do not enhance using contrast media, often displaying a visible scolex that appears as an opaque 2 to 3 mm nodule (Garcia, Gonzalez and others 2003). Inflamed (colloidal) viable cysts display edema and enhancement in the surrounding tissue. Granulomatous cysts show enhancement with the

disappearance of cystic fluid and appear opaque and dense, signaling degeneration, whereas completely calcified nodules are indicative of residual disease. Enhancing cystic lesions can easily be misdiagnosed because tuberculomas, abscesses and tumors may produce similar images (Garcia and Del Brutto 2000; Del Brutto and others 2001; Carpio 2002).

Diagnostic criteria for NCC (Del Brutto and others 2001) are based on histological, neuroimaging, immunological and epidemiological data. Absolute criteria for identification of *T. solium* are either by direct evidence through biopsy (CNS) or funduscopy (eye), or by visualization of scoleces on CT or MRI. All other criteria are ordered in descending fashion according to their diagnostic strengths into major, minor and epidemiological categories. If no absolute criteria have been met, evidence of two major, one minor and one epidemiological criterion are required to give definitive diagnosis of NCC. A probable diagnosis of NCC is made when patients exhibit a combination of three or four criteria of lesser diagnostic strength (Del Brutto and others 2001). One of the major criteria for NCC diagnosis is a positive immunological test for anticysticercal antibodies. Although several immunological assays for cysticercosis have been produced, the most reliable is the enzyme-linked immunoelectrotransfer blot (EITB) (Tsang and others 1989). The EITB is 100% specific and 98% sensitive for clinically confirmed cases of NCC (Brand and Tsang 1989; Tsang and others 1989, 1991) and has been recognized as the serological assay of choice for the detection of cysticercosis in humans and pigs by the World Health Organization/Pan American Health Organization (WHO/PAHO) in 1995 (PAHO 1997). The EITB is frequently used as a confirmatory test in cases where an absolute criterion for NCC diagnosis is not met.

Patients with confirmed cases of NCC who experience seizures are treated with antiepileptics concurrently with steroids to reduce inflammation. Patients who experience

hydroencephaly or hypertension are often managed with ventricular shunts to ease intracranial pressure (Loyo and others 1980). Surgical resection may be indicated for patients having ventricular cysts or mass lesions that cause either CSF obstruction or spinal cord compression (Loyo and others 1980; Stern 1981; Zee and others 1984; Garcia and Del Brutto 2000; Citow and others 2002). Depending on individual diagnosis, the patient may also receive an antiparasitic drug, either praziquantel (Botero and Castano 1982) or albendazole (Escobedo and others 1987). Generally longer (8 to 15 day), low-dose treatment schemes are more effective for patients with multiple cysts, whereas shorter (1 to 3 day), high-dose treatments are effective for patients with single lesions (Garcia, Gonzalez and others 2003). Factors considered when determining whether these medications would be of benefit are based on the number, viability, and location of cysts, as antiparasitic chemotherapy is known to cause or exacerbate inflammation (Moodley and Moosa 1989; Garcia, Gilman, Horton and others 1997; Salinas and Prasad 1999; Garcia and others 2002). Until recently, there were actually two schools of thought regarding the benefits of antiparasitic treatment. While some clinicians believed that the risks associated with chemotherapy were unwarranted because cysticerci eventually die and resolve on their own (Padma and others 1994; Kramer 1995; Carpio and others 1995; Caplan 1997), others found that antiparasitic treatment led to improved clinical outcome (Sotelo and others 1984; Vazquez and Sotelo 1992; Del Brutto 1995; Baranwal and others 1998; Garcia and others 2004). In 2002, however, a consortium of neurologists and other experts in the study of NCC established consensus guidelines for patient management (Garcia and others 2002). The consortium reached a consensus in favor of antiparasitic treatment in cases of moderate (5 to 100 cysts) parenchymal infections and subarachnoid racemose cysts, and a consensus against antiparasitic treatment in cases of encephalitis and hydroencephaly, as well as residual, calcified cysts. In all other

diagnostic scenarios, no consensus could be reached, so a series of alternative treatment recommendations were given. More recently, a longitudinal, double blind, placebo-controlled study (Garcia and others 2004) has shown that antiparasitic treatment with the drug albendazole significantly reduced the occurrence of generalized seizures. This study may warrant further revisions to the current consensus guidelines regarding antiparasitic chemotherapy.

The primary method for the detection of taeniasis is by direct examination of stool for eggs and proglottids. Often, samples must be collected over a period of several days, as proglottid shedding is intermittent (Schmidt and others 1996). Because eggs of *Taenia saginata* and *T. solium* cannot be differentiated by visualization, gravid proglottids must be present to make a definitive diagnosis (Wilkins and others 2002). The two species are identified according to the number of branches leading from the median stem of the uterus; *T. solium* has 7 to 13 of these branches, whereas *T. saginata* has 15 to 20 (Schmidt and others 1996). Unfortunately, visualization of eggs and proglottids in successive stool samples is cumbersome and prone to error (Hall and others 1981). However, detection of *Taenia* parasite coproantigen in the feces can be accomplished using a rapid, enzyme-linked immunosorbent assay (ELISA) that utilizes polyclonal rabbit antibodies raised against adult worm somatic (Allan and others 1990) or excretory/secretory antigens (Wilkins and others 1999). Although the coproantigen assay is not species specific, it requires only one stool sample, and is 2.6 times more sensitive than microscopic examination (Allan, Velasquez-Tohom, Torres-Alvarez and others 1996), as well as 99% specific for *Taenia* genera infections (Allan and others 1992, Allan, Velasquez-Tohom, Torres-Alvarez and others 1996). However, in our experience, the coproantigen assay has proven highly sensitive to *T. solium* infections and does not detect *T. saginata* infections nearly as well. Positive coproantigen immunoassays become negative one week after successful

treatment with the antiparasitic drug, niclosomide (Allan and others 1990, Deplazes and others 1991).

EPIDEMIOLOGY AND GLOBAL SIGNIFICANCE

Neurocysticercosis is now considered the most important neurological disease of parasitic origin in humans (WHO 2002). The disease is prevalent in rural populations of developing nations where pigs dwell in close proximity to humans. Endemic areas include Latin America, China, India, Southeast Asia and Sub-Saharan Africa (WHO 2002). It is estimated that 75 million people in Latin America live in endemic areas, about 400,000 of whom develop symptomatic disease (Bern and others 1999). Serological data from surveys performed in rural communities of Mexico, Bolivia and Peru show that prevalence of human cysticercosis in these areas is between 8 and 12% (Garcia and others 1991), yet up to 40% of seropositive individuals will become seronegative within one year, complicating prevalence estimates. Infected individuals may also remain asymptomatic for years, possibly throughout the duration of viable infection, leading to underreporting of infected households.

Epidemiological studies have shown that NCC is the leading cause of adult-onset epilepsy in endemic countries (Medina and others 1990; Garcia and others 1993; White 1997; Palacio and others 1998; Rajbhandari 2004) and accounts for up to 12% of all admissions to neurological hospitals in Peru (Garcia and others 1991). Epilepsy is the primary manifestation of NCC, accounting for roughly 70% of symptomatic cases (Del Brutto and others 2001). Persons with epilepsy may be unable to work because of frequent seizures, and are likely to sustain other injuries because of seizure activity (Wandra and others 2000). Furthermore, epileptics are often stigmatized in rural communities where disease etiology is poorly understood (Bharucha 2003;

Diop and others 2003). Because of this, persons suffering from symptomatic NCC are also likely to suffer serious personal, social and financial difficulties. To further compound the impact of disease, adequate clinical facilities with computer-based imaging equipment are seldom available in rural areas, so travel and extended stays away from family members are often requisite for those who seek medical attention.

Transmission of the disease is perpetuated by socioeconomic conditions, as pigs raised by individual households for sustenance or resale are allowed to forage freely, feeding on human refuse. Few pigs are taken to commercial abattoirs, and those that are receive only a perfunctory lingual examination for disease (Gonzalez and others 1990, 1997; Cysticercosis Working Group in Peru (CWGP) 1993). Often, pigs with known cysticercosis infections are sold on the black market to prevent economic loss (Gonzalez and others 1990, 1997; Garcia, Gilman and others 2003). The absence of health care infrastructure and lack of education regarding preventative personal hygiene also greatly contribute to transmission of NCC in these areas. Inadequate latrine facilities and sewage disposal, as well as the use wastewater for irrigation of crops, ensure host availability for the parasite (Pawlowski 2002). Unchecked transmission of NCC further impacts the economies of endemic countries by straining limited health care budgets, and negatively impacting the pork industry. Several years ago, it was estimated that hospital admission cases in Mexico cost US\$15 million annually (Flisser 1988). This figure does not address any patient management costs, such as drug therapy or subsequent hospital stays. The pork industry suffers even greater losses due to *T. solium* transmission, and resulted in the loss of more than half of the national investment in swine production in Mexico, with an overall loss of US\$164 million for Latin America (Murrell 1991).

CONTROL AND ERADICATION

The International Task Force for Disease Eradication declared *Taenia solium* a potentially eradicable parasite in 1993 (Centers for Disease Control (CDC) 1993; WHO 2002), given that humans and domestic pigs are its only transmissible reservoirs of infection. With strategic use of antiparasitic treatment of human taeniasis carriers and cysticercotic pigs, interruption of the *T. solium* life cycle is feasible (WHO 2002). The task force further stated that research efforts should be focused on the development of a “simpler” diagnostic assay to detect infected pigs and humans in order to realize the goals of disease control and eradication (CDC 1993).

Prior to development of the EITB in 1989 (Tsang and others 1989), neurological imaging was the only definitive means of diagnosis, as immunoassays available before that time were of low sensitivity and cross-reactive with other parasitic infections. Although the EITB has been instrumental in disease surveillance of both humans and pigs in isolated, endemic populations, it relies on native cyst antigens that will become less available as disease transmission is effectively controlled. Because the immunoblot format of the EITB is not well-suited to the high-throughput capacity that will be required to monitor infection rates within a sizable geographic region, a rapid assay format must be developed. Several of the EITB native glycoprotein antigens have been sequenced, cloned and reproduced either synthetically or recombinantly (Greene and others 1999, 2000; Hancock and others 2003, 2004). Assay development using these highly sensitive and specific bioengineered protein antigens will allow for high capacity field-testing of both pigs and humans (Handali and others 2004; Bueno and others 2005). Because cysticercosis infections tend to cluster around tapeworm carriers (Allan, Velasquez-Tohom, Garcia-Noval and others 1996; Garcia, Gilman and others 2003), surveillance using these high-throughput assays will assist in locating taeniasis cases. Identification of

tapeworm carriers may be accomplished using either the coproantigen immunoassay (Allan and others 1990), or preferably, an ELISA designed with recombinant, *T. solium*-specific, diagnostic adult worm antigens (Levine and others 2004). Surveillance programs using these immunodiagnostic assays will identify infected pigs and tapeworm carriers so that appropriate antihelminth treatments can be dispensed to abrogate the *Taenia solium* life cycle. Although other interventions, such as education, sanitation, and fortification of health infrastructures in endemic regions will be necessary for the success of eradication efforts, immunoassay surveillance is a necessary first step.

CHAPTER 2

SERODIAGNOSIS OF NEUROCYSTICERCOSIS USING SYNTHETIC 8-KDA PROTEINS;
COMPARISON OF ASSAY FORMATS¹

¹ Scheel CM, Khan A, Hancock K, Garcia HH, Gonzalez AE, Gilman RH, Tsang VCW.
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INTRODUCTION

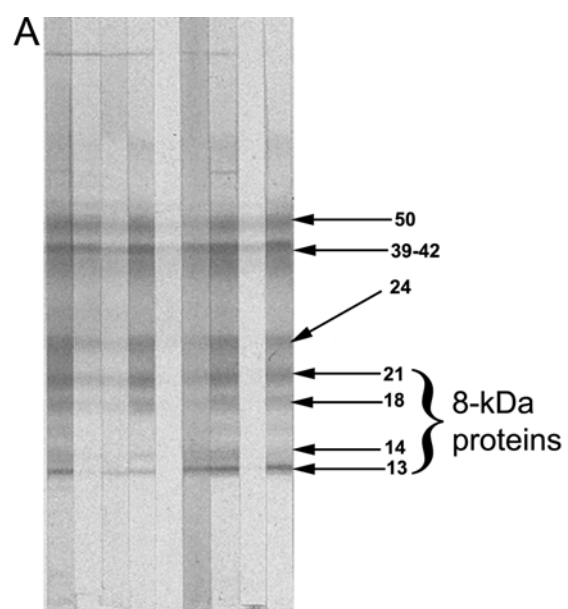
The World Health Organization has recently classified neurocysticercosis (NCC) as the most important neurological disease of parasitic origin in humans (WHO 2002). Neurocysticercosis is a major cause of adult-onset epilepsy in areas where the pork tapeworm *T. solium* is endemic (Medina and others 1990; Garcia, Gilman, Tsang and others 1997; White 1997; Palacio and others 1998; Rajbhandari 2004). 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, hydroencephaly, 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 neurological 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 serological assay. The enzyme-linked immunoelectrotransfer blot (EITB), a western blot assay developed in 1989 that is 98% sensitive and 100% specific for detecting pathologically confirmed cases of NCC (Brand and Tsang 1989; Tsang and others 1989, 1991) was recognized as the serological assay of choice for the detection of cysticercosis in humans and pigs by the WHO/PAHO in 1995 (PAHO 1997). To produce the assay, cysts are excised from porcine tissue, homogenized, and partially purified on a lentil-lectin column. The resulting lentil-lectin glycoproteins (LLGP) 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 at 13, 14, 18, 21, 24, 39-42, and 50 kDa (Figure 2.1) (Brand and Tsang 1989; Tsang and others 1989). 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 cysticercosis antibodies 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, as it lends itself to high-throughput diagnostic applications. Previous attempts to utilize LLGP in an ELISA format have failed, as it contains irrelevant proteins that bind to polystyrene with a higher affinity than any of the seven *T. solium*-specific antigens. In order 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 (Greene and others 1999, 2000; Hancock and others 2003, 2004). The LLGP antigens at 13, 14, 18 and 21-kDa (Figure 2.1) have been identified as belonging to a large, closely related group of proteins representing four distinct phylogenetic clades, the 8-kDa antigen family (Greene and others 2000; Hancock and others 2003). Previous results showed that 48 to 82% of patients tested reacted in the EITB with these antigens (Tsang and others 1989). Four chemically synthesized representative TS (*Taenia solium*) antigens from each of the four clades, TS14, TS18var1, TSRS1, and TSRS2var1, were assayed individually by ELISA and western blot for immunoreactivity against large cohorts of sera from clinically

Figure 2.1. Example of the enzyme-linked immunoelectrotransfer blot (EITB), showing the seven antigenic bands found in partially purified lentil-lectin glycoproteins (LLGP). Each strip was probed with serum from a neurocysticercosis (NCC) clinical case. The LLGP antigenic bands at 13, 14, 18, and 21 kDa are post-translationally modified 8-kDa proteins.



defined neurocysticercosis patients, healthy individuals who had not traveled outside of the US, and persons infected with other parasitic diseases from areas without cysticercosis.

MATERIALS AND METHODS

Defined Serum Collections. Defined cysticercosis serum samples ($n = 377$) were collected at the Nacional de Ciencias Neurológicas, Lima, Perú, 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 in Del Brutto OH and others 2001. Patient samples were collected in compliance with protocols approved by the ethical review boards of all institutions concerned (CDC, Universidad Peruana Cayetano Heredia). All patients involved in this study provided written 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, as single lesions are not clear indicators of NCC (Wilson and others 1991; Garcia, Gilman, Tsang and others 1997; Del Brutto and others 2001). 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 (data not shown). To define the specificity of the 8-kDa antigens for cysticercosis, serum samples from healthy individuals who had not traveled outside of the United States (normal human sera, NHS) ($n = 146$), and sera from persons infected with other parasitic diseases from countries where NCC is non-endemic ($n = 162$) were tested. The infected serum used to test for specificity were from

confirmed cases of *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), *Schistosoma mansoni* (36), *Trichinella* spp. (16), and *Taenia saginata* (14) infections. 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-kDa Antigens. The full-length, 8-kDa proteins were chemically synthesized without their signal sequences by commercial manufacturers at a purity of $\geq 95\%$ as follows: TS14 (AF082829), (Gryphon Sciences, San Francisco, CA and AnaSpec Inc., San Jose, CA); TS18var1 (AF098073), (AnaSpec); TSRS1 (AF082830) (AnaSpec); TSRS2var1 (AF356343), (SynPep Corp., Dublin, CA and AnaSpec). All of the synthetic 8-kDa mature protein sequences are 66 amino acids in length, with the exception of TS18var1, which is 67 amino acids long (Table 2.1). 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 (ELISA). The quantitative determination of patient seroreactivity to the 8-kDa proteins was determined by the FAST-ELISA method described previously (Hancock and Tsang 1986). Briefly, lyophilized 8-kDa antigens were solubilized in 0.05 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) pH 7.0 /0.1 M NaCl/2 mM dithiothreitol (DTT) and quantitated by 280 nm 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

Table 2.1. **Mature protein sequences of the full-length, *Taenia solium* 8-kDa antigens.** Chemically synthesized 8-kDa antigens, TS14, TS18var1, TSRS1, and TSRS2var1, were evaluated in enzyme-linked immunosorbent (ELISA) and enzyme-linked immunoelectrotransfer blot (EITB) serodetection assays (Greene and others 2000; Hancock and others 2003).

Protein Name	GenBank Accession no.	Sequence
TS14	AF082829	EKNKPKDVANSTKKGIEYVHEFFHEDPIGKQIAQLAKEWKEAMLEDKKGKIRTSLVEHCKGPKKKTA
TS18var1	AF098073	EKIKPKCDTNSTKKEIEYIHNWFFHDDPIGKQIAQLAKNWNENTVQEAKGEIRASLAEYCRGLKNKTA
TSRS1	AF082830	EETKPEDVVKNIKKGMEVVYKFFYEDPLGKKIAQLAKDWKEAMLEARSKVRASLAEYIRGLKNEAA
TSRS2var1	AF356343	EKNKTDDVGKSIKNWIEFVHRFFYEDPIGKQIAQLAKDWNETAPEARCKVRALLAENRRGLKNKTA

was diluted in phosphate buffered saline (PBS) pH 7.2 to a concentration of 0.01 mg/mL, then sensitized to the polystyrene sticks of NUNC-TSP transferable solid phase screening system lids (cat# 445497, Nunc[™], Nalge Nunc International, Denmark) from the corresponding 96-well flat bottom incubation plates (cat # 269620, Nunc[™]). The sticks were sensitized for 2 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, cysticercosis-positive patient serum titrated to represent 10 unit values of seroreactivity (Hancock and Tsang 1986). When tested against the 8-kDa antigens, the seroreactivity of the pooled serum used in the standard curve was found to vary in intensity between antigens. In order 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 (Bradford 1976). The solubilized hIgG was used to construct a 10-point standard curve by dilution into PBS/0.01% bovine serum albumin (BSA) Fraction V/0.1% sodium azide with IgG concentration values ranging from 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.

ELISA 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[®]) using a pressurized garden sprayer. Washes were performed in this manner between all subsequent reagent incubation steps. All reagents were dispensed at 150 µL/well,

and incubated at room temperature for 5 min 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 employed on each plate to insure intra-assay integrity. Goat anti-human heavy and light chain IgG conjugated to horseradish peroxidase (GAHG-POD) (Tsang and others 1995), 1.86 mg/mL, was diluted to a working concentration of 1:1000 in wash buffer and used to detect reactive antibody, followed by development with SureBlue[™] TMB Microwell Peroxidase Substrate (1 component) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Absorbance at 650 nm was read by a THERMOmax microplate reader using SoftMax Pro 4.7.1 software (Molecular Devices Corp., Sunnyvale, CA). Interassay coefficient of variance (CV) for the positive controls was calculated to insure validity of the assay. Plates with a positive control unit value CV that was greater than 10% outside of the interassay mean were repeated.

Enzyme-linked Immunoelctrotransfer Blot (EITB). 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. TSRS1 contains no cysteines, so no DTT was included in the solubilization buffer. Proteins were quantitated by UV absorption as above. The EITB was performed as indicated in Tsang (1989). All defined cysticercosis, other parasitic diseases, and normal human serum samples were tested for seroreactivity against all four of the 8-kDa antigens. Assays for each antigen were repeated by a second party in order 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 (Schleicher & Schuell, cat. # 10448015). All reagents were dispensed at 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 for 5 min, 4 times in wash buffer using the Accutran™ automatic strip washer (Schleicher & Schuell). GAHG-POD conjugate was diluted 1:8000 in 0.05 M Tris/0.5 M NaCl/0.3% Tween 20®, and the strips were incubated for 1 hour at room temperature with gentle rocking. The antigen strips were then washed for 5 min, 3 times with wash buffer and 2 times in PBS, before a 10-minute incubation in 3,3'-diaminobenzadine (DAB) substrate. After substrate development, strips were rinsed 10 times in dH₂O, dried, and aligned for analysis.

In order 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 to EITB, an NCC patient with very low seroreactivity to EITB, a patient from a cysticercosis non-endemic country infected with a different parasite, and an NHS serum sample (data not shown). The ideal gel loading concentrations (mg/mL) were: TS14 (0.005), TS18var1 (0.001), TSRS1 (0.020), and TSRS2var1 (0.005). 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 form non-specific protein/protein interactions in the blot format with some frequency. 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

ELISA. 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 ($n = 206$), the normal human sera (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 2.2). Overall assay efficacy, as measured by Youden's J Index ($[\text{Sensitivity} + \text{Specificity}] - 1$) (Youden 1950) 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 here.

Each of the four 8-kDa antigens proved to have 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-kDa antigens and their respective cutoff values are shown in Figure 2.2. This evaluation study was repeated with highly similar results, so it was decided to discontinue testing in this assay format.

EITB. With the exception of TS14, all of the protein antigens performed better in the EITB format than in ELISA, as shown by increases in Youden's J Index, which was again calculated using only those serum samples from patients with multiple viable and racemose cysts ($n = 107$) against the serum from persons with other parasitic diseases from NCC non-endemic countries ($n = 162$) and NHS samples ($n = 146$) (Table 2.3, Table 2.2). The J Index values for all four antigens were highly similar to those shown in Table 2.3 when calculated using only those NCC and control serum used for evaluation of the 8-kDa ELISA ($n = 382$) (Table 2.2), so the results of the larger cohorts are described here. TSRS1 showed the greatest increase in sensitivity to

Table 2.2. Performance of four *Taenia solium* 8-kDa antigens in an enzyme-linked immunosorbent assay (ELISA) format when tested against sera from clinically defined neurocysticercosis patients showing various stages of disease progression ($n = 206$). Serum samples from persons infected with other parasitic diseases from countries where cysticercosis is non-endemic ($n = 79$), and healthy individuals who have not traveled outside of the United States ($n = 97$) were used to determine antigen specificity.

Antigen (mg/mL)	% Sensitivity				% Specificity ($n = 176$)	Youden's J Index
	≥ 2 viable and racemose cysts ($n = 73$)	Single viable cyst ($n = 16$)	Degenerating cysts ($n = 43$)	Calcified cysts ($n = 74$)		
TS14 (0.01)	90	50	42	30	85	0.756
TS18var1 (0.01)	95	44	56	49	85	0.791
TSRS1 (0.01)	77	50	33	20	93	0.699
TSRS2var1 (0.01)	86	44	35	31	86	0.721

Figure 2.2. **Performance of four *Taenia solium* 8-kDa antigens in the enzyme-linked immunosorbent assay (ELISA) format.** The plots show serum antibody reactivity of neurocysticercosis patients (NCC) having either ≥ 2 viable or racemose cysts ($n = 73$), patients with other parasitic diseases (Others) ($n = 79$), and normal human sera (NHS) ($n = 97$). Cutoff points for **a**, TS14, **b**, Ts18var1, **c**, TSRS1, and **d**, TSRS2var1, were determined using Youden's J Index ($[\text{Sensitivity} + \text{Specificity}] - 1$), and are shown by the horizontal line with the corresponding unit value at right (Youden 1950). Units of activity in all plots are based on the standard curve and are shown in \log_{10} -scale format.

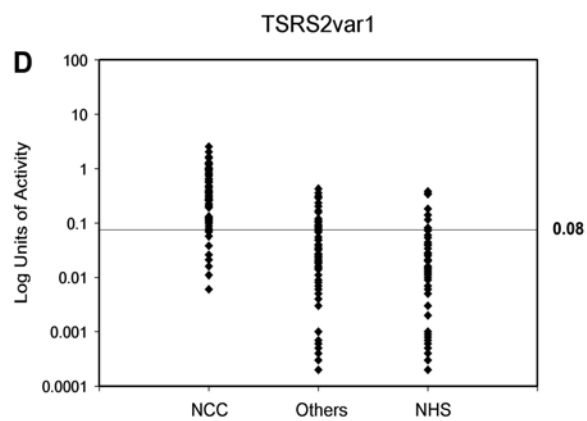
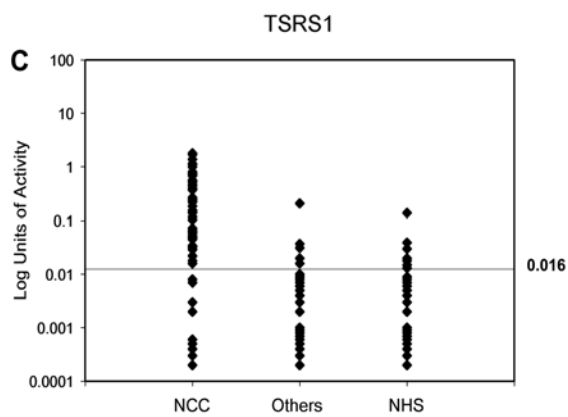
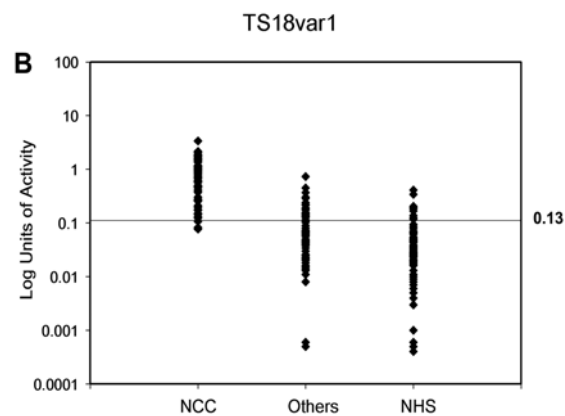
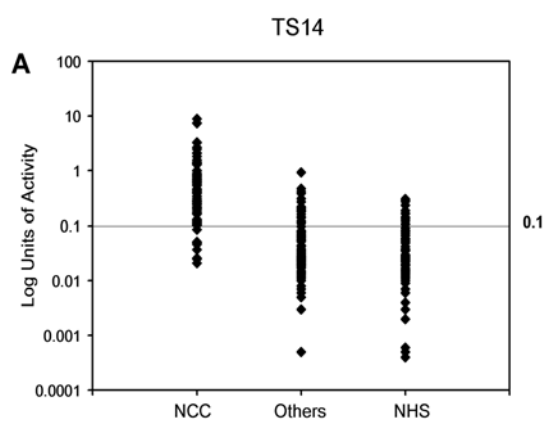


Table 2.3. Performance of four *Taenia solium* 8-kDa antigens in an enzyme-linked immunoelectrotransfer blot (EITB) format. All four antigens were tested against sera from clinically defined neurocysticercosis (NCC) patients showing various stages of disease progression ($n = 307$) to determine their sensitivity. Serum samples from persons infected with other parasitic diseases from countries where cysticercosis is non-endemic ($n = 162$), and healthy individuals who have not traveled outside of the United States ($n = 146$) were used to determine antigen specificity. The Youden's J Index values (Youden 1950) for all four antigens were similar when calculated using the same NCC patient and control serum samples used in 8-kDa-ELISA ($n = 382$), so results of the larger serum cohorts are shown here.

Antigen (mg/mL/mm)	% Sensitivity				% Specificity ($n = 308$)	Youden's J Index
	≥ 2 viable and racemose cysts ($n = 107$)	Single viable cysts ($n = 20$)	Degenerating cysts ($n = 66$)	Calcified cysts ($n = 114$)		
TS14 (0.005)	94	70	55	63	76	0.703
TS18var1 (0.001)	97	35	44	33	100	0.972
TSRS1 (0.020)	96	85	61	47	98	0.943
TSRS2var1 (0.005)	82	40	38	29	98	0.800

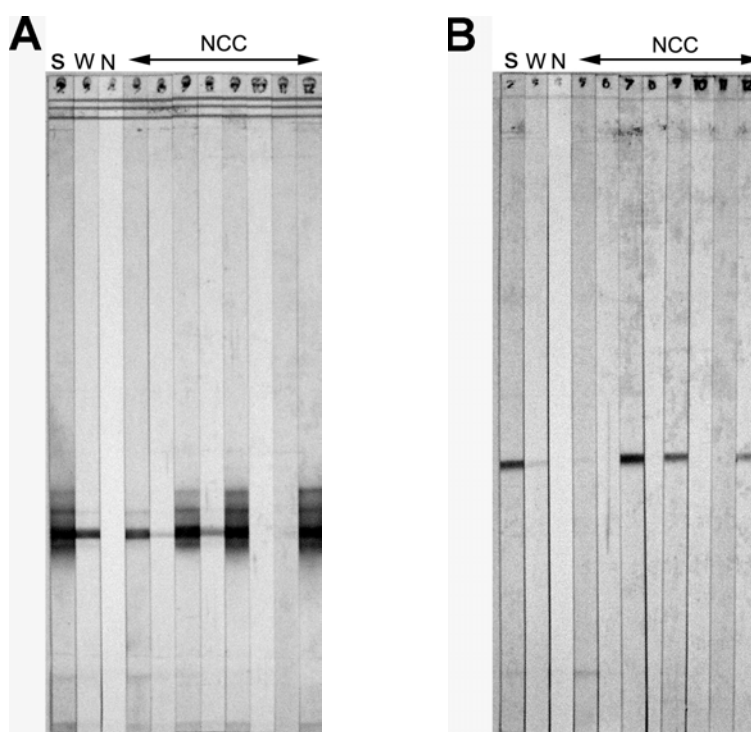
multiple viable and racemose cysts (from 77 to 96%), whereas all other proteins showed lesser change in this disease category ($\pm 5\%$). Likewise, TSRS1 showed the greatest improvement overall in detecting all other disease states, increasing in sensitivity by 35% for single cysts, 28% for degenerating 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%, lower than that of the ELISA format (85%). Except for TS14, all of the protein antigens showed improvement in specificity. Only TSRS1 improved in sensitivity for all disease categories as well as showing increased specificity over the ELISA format.

Both TSRS1 and TS18var1 had Youden's J Index values over 0.9 in the EITB format, which is indicative of an efficient assay (Youden 1950). 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 2.3A). Weak positive NCC patient samples are harder to distinguish on the TS18var1 blot (Figure 2.3B), 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, native EITB (PAHO 1997). The development of synthetic and recombinant LLGP antigens has afforded the opportunity to assess the feasibility of supplanting the native EITB as the standard serological assay for cysticercosis confirmation. The results of the TSRS1-EITB indicate that native antigen is not superior to synthetic for NCC antibody detection in this assay format, showing a sensitivity of 96% and a specificity of 98%, comparable to that of LLGP-EITB (Table 2.3). A distinct advantage of TSRS1-EITB is that only one

Figure 2.3. **Comparison of western blots of two *Taenia solium* 8-kDa antigens tested against neurocysticercosis (NCC) patient sera.** Blot strips from both **A**, TSRS1 and **B**, TS18var1 were probed with a strong NCC-positive control serum (S), a weak positive control serum (W), and a control strip with no antibody added (N). The remainder of the strips (between arrows) were probed with the same set of NCC patient serum.



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 2.3). 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 (data not shown). The ultra-low concentration of this antigen on EITB is problematic in two ways. First, the positive cases are not as clearly defined, often resulting in very faint bands (Figure 2.3B). Second, the consistent reproducibility of this assay is not assured at such a low concentration. TSRS1, on the other hand, 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 (data not shown).

The secondary objective of this study was to develop a quantitative confirmatory assay for cysticercosis that may impart information regarding patient disease status, as well as lend itself to a high-throughput assay format. Unfortunately, direct coating of the 8-kDa 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 TS18var1 ELISA had the highest Youden's J Index value (0.791), its specificity for NCC cases is too low (85%) for accurate interpretation in clinical settings.

Initial ELISA-assay evaluation studies using biotinylated 8-kDa antigens, sensitized to streptavidin-coated polystyrene, yielded promising results (Hancock and others 2003). When

biotinylated TS18var1 was further tested in ELISA against a larger cohort of sera (202 samples, total), 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-kDa antigens in the ELISA format to date.

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 (Hernandez and others 2000; Hancock and others 2003; Fleury, Beltran and others 2003). Although all of these published trials employed their respective peptide antigens in an ELISA format, only one, the biotinylated 8-kDa antigen trial, was a serodetection assay (Hancock and others 2003). The others relied on extraction of patient cerebrospinal fluid (CSF) 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-kDa 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 (Butler 2004). Future seroassay development with 8-kDa peptides will include direct application of 8-kDa synthetic antigens to nitrocellulose, and an ELISA in which the antigen/antibody binding occurs in solution.

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CHAPTER 3

DEVELOPMENT OF A NORMAL HUMAN IMMUNOGLOBULIN G STANDARD CURVE
FOR ENZYME-LINKED IMMUNOSORBENT ASSAY: USE FOR COMPARISON OF
ANTIGEN EFFICACY²

² Scheel CM, Handali S, Bueno EC, Khan A, Hancock K, Gonzalez AE, Garcia HH, Gilman RH, Tsang VCW. Submitted to *Journal of Immunoassay and Immunochemistry*.

INTRODUCTION

The indirect enzyme-linked immunosorbent assay (ELISA) is currently the most widely used method of detecting and quantifying humoral immune responses. In the past, diagnostic antigens used to develop such assays were derived from crude extracts of pathogens that often contained irrelevant, cross-reactive components. Recent advances in recombinant protein technology have provided a means of circumventing the problems associated with non-specific reactivity in crude antigen ELISAs; development of indirect ELISAs based entirely on recombinant protein antigens is now common practice. The most desirable recombinant ELISA assay format will require only one antigen to detect specific antibody reactivity in an infected population. Often, however, native lysates are comprised of several antigens that elicit sensitive and specific humoral responses in diagnostic assays. In order to determine which native antigen is the most suitable for large-scale assay development, it may be necessary to produce several recombinant antigens for assay against infected material.

Ideally, an internal standard reference curve should be included in each microwell ELISA plate to control for fluctuations in optical density (OD) caused by slight variations in reagent temperature, volume, pH or formulation. The standard curve is assigned arbitrary unit values that remain constant regardless of these deviations in OD. Most often, the standard curve is comprised of pooled immunoreactive samples collected from several donors with proven infections. Because of the rarity of some conditions and diseases, these samples may not be available in sufficient quantities to develop such a standard curve. Even if the immunoreactive material is readily available, it is unlikely that the sources for individual components of the pool will be available indefinitely. Furthermore, because immune response to a given antigen will likely vary between individuals, the pooled immunoreactive material may be skewed to

recognize one antigen over another, and may not be representative of the target population. An unbiased, universal standard curve is necessary to compare the specific activity of several antigens on the same reactivity scale.

We have developed a standard curve for indirect ELISA using normal human immunoglobulin G (hIgG) that allows us to compare the specific activity of many antigens on a universal scale to determine the most efficient antigen(s) for large scale assay development. Preparation of the curve is simple; hIgG is solubilized, quantified, and titered into several dilutions to represent a reliable curve that falls within the limits of spectrophotometric detection. The chosen dilutions can then be prepared in larger batches and stably stored at 5 °C for up to one year. The hIgG standard curve has proven to be a critical tool for the comparison of several antigens slated for large-scale assay development in this laboratory.

MATERIALS AND METHODS

Normal Human Immunoglobulin G. Normal, human immunoglobulin G (hIgG) was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and ionic exchange chromatography to homogeneity (Tsang and others 1984), lyophilized, and frozen in the vapor phase of liquid nitrogen. The hIgG was resolubilized in degassed phosphate-buffered saline (PBS) pH 7.2/ 0.1% sodium azide and mixed end over end for 15 min. The solubilized hIgG was then quantitated using the standard Bradford Assay (Bradford 1976).

Preparation of hIgG Standard Curve. The hIgG solution was diluted in PBS pH 7.2/0.1% sodium azide/0.01% bovine serum albumin (BSA) Fraction V to a working concentration of 20 $\mu\text{g/mL}$. A pilot curve of ten standards (10 mL each) was prepared in the following concentrations ($\mu\text{g/mL}$): 0, 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, and 1.0. The standards were

assigned unit values of reactivity that corresponded to their respective concentrations, shown above, with the maximum value of 1.0.

Enzyme-Linked Immunosorbent Assay (ELISA) Evaluation of hIgG Standard Curve. The FAST-ELISA method used to evaluate the curve has been described previously (Hancock and Tsang 1986). All reagents were dispensed at 150 μ L/well. Briefly, the ten standards were dispensed in 96-well flat bottom incubation plates (cat # 269620, NuncTM, Nalge Nunc International, Denmark) and adsorbed to the polystyrene sticks on lids of the NUNC-TSP transferable solid phase screening system (cat# 445497, NuncTM) at room temperature for 2 hours with gentle shaking. This step is hereafter referred to as sensitization. The sticks were then rinsed with a wash buffer of PBS/0.3% Tween[®] 20 (polyoxyethylene sorbitan monolaureate, Calbiochem[®], San Diego, CA) using a pressurized garden sprayer. Goat anti-human heavy and light chain IgG conjugated to horseradish peroxidase (GAHG-POD) (Tsang and others 1995) was diluted in wash buffer to a non-rate-limiting concentration (previously determined by titration) and used to detect the bound antibodies. The GAHG-POD was incubated for 5 min with gentle shaking, and sticks were rinsed with wash buffer as before, followed by substrate development using SureBlueTM TMB Microwell Peroxidase Substrate (1 component) (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Absorbance at 650 nm was read by a THERMOmax microplate reader using SoftMax Pro 4.7.1 software (Molecular Devices Corp., Sunnyvale, CA). All units of the curve were tested in triplicate two times to ensure that the standard values were representative of a comprehensive curve that fell within the quantifiable absorbance range of the spectrophotometer.

Antigen Comparison using the hIgG Curve. A large batch of the hIgG curve was prepared in 50 mL quantities for each standard. Vials stored at 5 °C were later assayed against freshly

prepared hIgG curve to determine shelf life. For each assay ($n = 92$), the standard curve was dispensed into wells along the top row of a Nunc 96-well plate. The remaining wells of the plate were loaded with repeats of one of four different synthetic antigens derived from purified *Taenia solium* cyst homogenate, TS14, TS18var1, TSRS1 and TSRS2var1, at a concentration of 10 $\mu\text{L/mL}$ each (Greene and others 1999, 2000; Hancock and others 2003; Scheel and others, in press). Sticks were sensitized with hIgG or antigen for 2 hours with gentle shaking, and then rinsed with wash buffer. The hIgG-bound sticks were then incubated in wash buffer; while antigen-bound sticks were incubated in test antibody diluted 1:100 in wash buffer/5% nonfat dry milk. All reagents were dispensed at 150 $\mu\text{L/well}$. Conjugate and substrate incubations were as described above, with a final reading at 650 nm. This procedure is also described in Bueno (2005). A positive control serum sample was used on all assay plates. Interassay coefficient of variance (CV) for the positive control was calculated to ensure validity of the assay. Plates showing a positive control value with a CV of greater than 10% from the inter-assay mean were repeated. Antigen efficacy was determined using Youden's J Index $([\text{Sensitivity} + \text{Specificity}] - 1)$ (Youden 1950).

Determination of Shelf Life. Shelf life was determined by storing a freshly prepared hIgG standard curve at an elevated temperature to accelerate the aging process (Kennon 1964; Porterfield and Capone 1984; Connors and others 1986; Anderson and Scott 1991). A new batch of fourteen hIgG standards (0, 0.01, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1, 1.5, and 2.0 $\mu\text{g/mL}$) was prepared using a 20 $\mu\text{g/mL}$ working solution as before. The standards were assigned unit values of reactivity that corresponded to their respective concentrations, shown above. After pilot testing, a large batch (30 mL) of each the fourteen hIgG standards was prepared and dispensed into amber glass vials. The vials were stored at 5 °C, and an elevated

temperature of 37 °C, representing an accelerated model for storage (Porterfield and Capone 1984). A portion of the 5 °C curve was used to sensitize several plates for dry storage at 5 °C and 37 °C. After a 2-hour sensitization, plates were air-dried for 1 hour, placed in light protected, covered containers, and stored.

The acceleration model used for aging is based on the bracket table method described in Porterfield and Capone (1984). The incubation times used to determine shelf life (Table 3.1) are based on the assumption that the activation energy (E_a) of hIgG is 20 kcal/mole, and are calculated using a modified version of the Arrhenius equation (Porterfield and Capone 1984). Aged samples were tested in duplicate for stability using FAST-ELISA as described above. All assay plates included a standard curve stored at 5 °C as a control measure. Test curves were read as both unknowns and standards. Long-term stability was also tested using the hIgG standard curve that was prepared 1.5 years earlier and used for *T. solium* antigen comparison (Scheel and others, in press). Inter-assay CVs of the points comprising the linear portion of the curve (unit values 0.1 to 2.0) were calculated for all standards and unknowns assayed.

RESULTS

Antigen Comparison using the hIgG Curve. The hIgG standard curve reactivity was consistent throughout the duration of the *T. solium* antigen comparison study (Scheel and others, in press) (data not shown). Although temperature variations did cause the OD of the curve to fluctuate slightly, the positive control in each plate ensured that the unit values by which antigen reactivity was measured remained consistent. Interassay CV analysis of the positive control showed that only 14 of the 92 antigen plates tested fell outside of the interassay mean by greater than 10%.

Table 3.1. **Storage time intervals at 37 °C for accelerated aging of the human immunoglobulin G (hIgG) standard curve.** The storage times listed at the elevated temperature are based on the bracket table method (Porterfield and Capone 1984), which assumes the activation energy (E_a) of a substance to be 20 kcal/mole, and is calculated by rearrangement of the Arrhenius equation.

Real Time Equivalent at Storage Temperature (5 °C)	Accelerated Aging Temperature (37 °C)
1d	--
1w	4h
1m	17.5h
3m	2d 4h
4m	2d 22h
5m	3d 15h
6m	4d 9h
8m	5d 19h
1y	1w 2d

h = hours, d = days, w = weeks, m = months, y = years

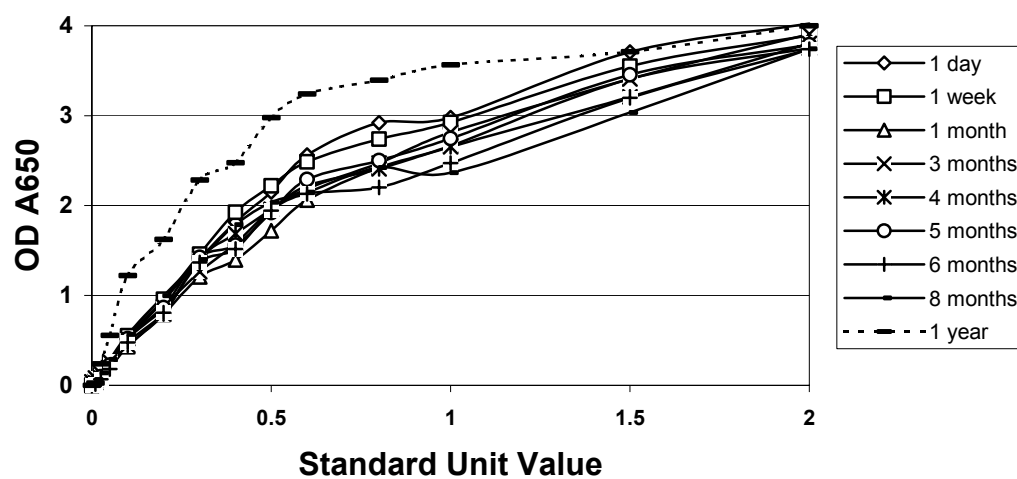
Determination of Shelf Life. Aged hIgG curve aliquots stored at 37 °C remained consistent in reactivity throughout the study, which was terminated at the real time equivalent of 1 year (Figure 3.1A). The interassay CV for the OD values of linear points on the curve (unit values 0.1 to 2.0) was calculated for the aged standard curve samples after 18 assays; each aged sample was tested in duplicate. Only one of the standard curves tested (1 year time point equivalent) deviated from the mean OD by greater than 10% at linear OD value points. All other standard curves had CVs of less than 10% for all equivalent linear points (units 0.1 to 2.0, $n = 18$ trials for each point), with a mean CV of 7.99%. Standard deviations between OD values for equivalent points of the curve ranged between ± 0.05 and ± 0.21 . An interassay CV of the assigned standard unit values of all of the time points tested, including the 1 year standard curve, was calculated at an average of 9.45%. This figure was very similar to the average CV calculated from the standard unit values of the 5 °C control curves (9.19%, $n = 18$) (data not shown). The hIgG standard curve also proved stable in real time aging conditions. The OD values of a standard curve stored at 5 °C for 1.5 years was compared with the mean OD data of four trials of the same curve tested in the first month of preparation (Figure 3.1B). The average CV calculated for the OD values comprising the linear portions (unit values 0.1 to 1.0) of both fresh and aged standard curves was 9.99%. Standard deviations of equivalent points of the curve ($n = 2$ trials for each point) ranged between ± 0.01 and ± 0.21).

Dry storage of the hIgG standard curve was unsuccessful, as the curve remained stable for only 1 day at both 5 °C and 37 °C, thereafter showing a decline in OD of approximately two-fold at 1 week (data not shown).

Figure 3.1. Stability of the human immunoglobulin G (hIgG) standard curve as shown by accelerated aging studies (A) and real time storage tests (B). An hIgG curve was stored at 37 °C to accelerate the aging process and tested at time points equivalent to real time aging at 5 °C (Porterfield and Capone 1984). The legend shows the real time equivalents of the accelerated aging time points tested (A). Real time aging of the hIgG standard curve showing a newly prepared curve (Mar-05) and a curve that was prepared 1.5 years earlier (Aug-03), both stored at 5 °C. Note that only nine standards are shown here, as the unit value “0.4” was masked in all early tests with this curve (B).

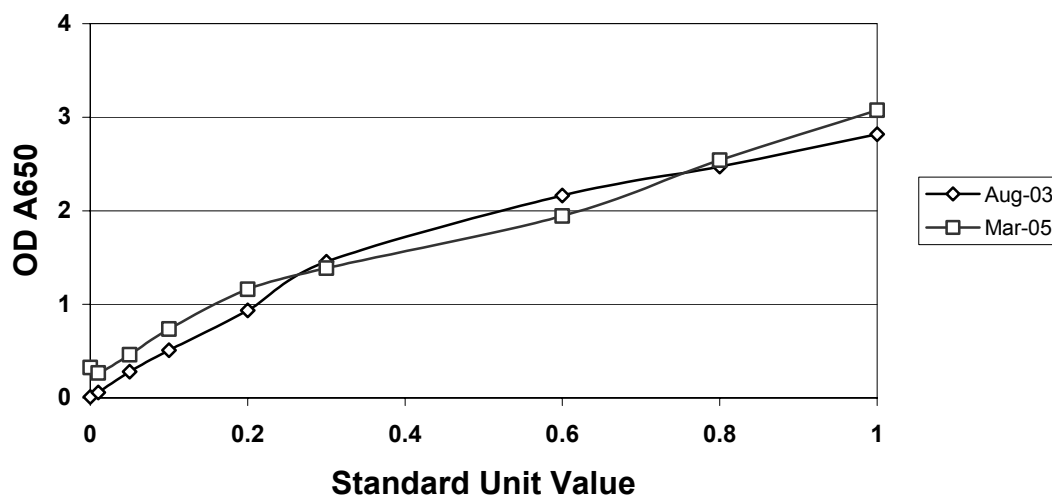
3.1A

**Aged Human Immunoglobulin G Standard
Curve Optical Density (OD) Values**



3.1B

**Stability of Human Immunoglobulin G
Standard Curve (1.5 years)**



DISCUSSION

The hIgG standard curve has played a pivotal role in the selection of diagnostic antigens worthy of large-scale assay development. When we initiated the evaluation of four synthetic *T. solium* antigens, TS14, TS18var1, TSRS1, and TSRS2var1 (Scheel and others, in press), we realized that the pooled, positive patient serum standard curve that we were using was immunologically biased. By using an hIgG standard curve, we were able to control for variances in all assay components, with the exception of immunological specificity of patient antibodies. Controlling for reagent, conjugate, and substrate variance is *a priori* for all assays that are comparing antigen efficacy. Additionally, the hIgG curve reflects assay activities that directly correspond to concentrations of hIgG in the analytes, and is independent of antigen-specific antibodies in infection sera.

In our evaluation of the *T. solium* antigens, we were able to eliminate potential diagnostic candidates from further development by utilizing a standard curve independent of immunological bias, saving both time and a great deal of expense. The hIgG standard curve can be also used to test individual sera for reactivity against a panel of antigens representing different infectious organisms to determine a relative level of reactivity to each. Use of this curve frees a diagnostic assay from the need for large quantities of positive sera from which to prepare a pooled standard curve to control for inter- and intra-assay variations. Instead, the standard curve is hIgG, and a positive serum sample is included as an unknown in the assay.

The highly purified hIgG used in this study was not from a commercial source, nor was the GAHG-POD conjugate. An attempt to repeat this study using commercially supplied hIgG and conjugate showed that much higher concentrations of hIgG (up to 20-fold) were needed to construct a standard curve that reached the upper limits of spectrophotometric detection

(personal communication, Jeff Priest). Because each standard curve is initially prepared as a test pilot batch, little time will be lost in determining the appropriate concentrations of hIgG needed to build a useful curve. It may be useful, however, to verify the purity of commercial hIgG before purchase, and to use hIgG from the same commercial source and lot when preparing new standard curves. For assays that may potentially involve IgM, IgA, or other subclasses of Igs, a standard curve prepared from ammonium sulfate or alcohol-precipitated serum protein fraction may also be considered. Alternatively, a mixture of purified Igs may be used to construct the standard curve. Albeit, the relevance of this issue is hard to ascertain, given that the 2nd enzyme-labeled antibody that we used here (GAHG-POD) possessed both heavy and light chain specificity and can detect all Ig subclasses equally.

It is unfortunate that the hIgG standard curve cannot survive dry storage, but it may be possible to retain curve sensitivity by storing pre-sensitized, wet plates (sealed) at 5 °C, although this has not yet been attempted. In this way, both the antigen and standard curve could be prepared in advance for ease and expediency of use. The hIgG curve should not be frozen as prepared, as a loss of reactivity may result. It is possible that the addition of glycerol to prevent freezing of the hIgG will allow for storage terms beyond those attempted in this study.

The hIgG standard curve for indirect ELISA is a useful method by which to compare the immunodiagnostic potential of any number of antigens on a universal scale. Reliance on pooled immunoreactive material for assay standard development and production is dangerous in two ways; it is unlikely to adequately represent the immune responses of the target population, and is of limited availability. The hIgG standard curve is inexpensive and simple to prepare, and will retain sensitivity for at least one year when stored at 5 °C.

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CHAPTER 4

CORRELATION OF DISEASE PROGRESSION WITH LONGITUDINAL
SEROREACTIVITY CHANGES IN NEUROCYSTICERCOSIS PATIENTS USING
CYSTICERCOSIS QUICKELISA^{TM3}

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INTRODUCTION

The current “gold standard” assay for serodiagnosis of cysticercosis is the enzyme-linked immunoelectrotransfer blot (EITB) (Tsang and others 1989). In an attempt to end reliance on native *T. solium* cyst material, while retaining the high sensitivity and specificity of the EITB, several lentil-lectin glycoprotein (LLGP) antigens have been synthetically or recombinantly reproduced (Greene and others 1999, 2000; Hancock and others 2003, 2004). These antigens have been applied to both enzyme-linked immunosorbent assay (ELISA) and western blot assay formats (Scheel and others, in press). Although the synthetic antigen TSRS1 was successful in matching the sensitivity and specificity of the EITB in a western blot format, the ELISA format did not perform as well as expected with any of the LLGP antigens.

While the TSRS1-EITB would allow us to effectively replace native antigen with synthetic, the assay format lacks quantitative capacity, is cumbersome, and is not well-suited to high-throughput applications. In order to address these issues, we partnered with a commercial immunoassay development laboratory, Immunetics, Incorporated. Immunetics, Inc. had recently developed successful ELISAs for both anthrax and Lyme disease (Marangoni and others 2005) using a patented format, the QuickELISA[™]. To develop the QuickELISA[™] for cysticercosis, Immunetics, Inc. was provided with the diagnostic candidate antigens that had shown the most potential in previous assay trials, synthetic TS18var1 and TSRS1 (Greene and others 1999, 2000; Hancock and others 2003), as well as a recombinant antigen, rGP50 (Hancock and others 2004). Evaluation of the new cysticercosis antigen QuickELISA[™] assays established their superiority over all of the previous assays we had developed due to their simple platform and effective performance.

The cysticercosis QuickELISA™ assays had not only proven their diagnostic capability, but also had the capacity to measure immunoreactivity to the disease in a quantitative fashion. The quantitative capability of these assays raised the prospect of learning more about how immune response relates to disease progression in cysticercosis. In the early stages of neurocysticercosis (NCC), metacestode cysts in the brain are biologically active and appear hyaline on brain images, often with small opaque scoleces in the center. As the cysts start to die, the host tissue surrounding the parasites becomes inflamed. These inflamed cysts are indicative of host responses that often coincide with definable pathologies, such as seizures or headaches. Under constant immune attack, the cysts eventually become granular and biologically inactive, and appear opaque in neuroimages. Eventually, the inflammation subsides and some cysts completely calcify. An important point to consider is that not all cysts go through the stages of progression discussed above. Many cysts, in fact, for indefinable reasons, are absorbed by the host and simply disappear from CT and MRI images. This can happen at any stage during the life cycle of the cyst.

Recently, a longitudinal, double blind, placebo-controlled study (Garcia and others 2004) had shown that antiparasitic treatment with the drug albendazole reduced the occurrence of generalized seizures. Longitudinal serum samples paired with corresponding computed tomography (CT) and/or magnetic resonance imaging (MRI) data from this study were used to evaluate the potential for monitoring cysticercosis disease progression.

MATERIALS AND METHODS

Defined Serum Collection. The NCC patient serum used in this study was collected at the Nacional de Ciencias Neurológicas, Lima, Perú, by Hector Garcia. This collection was acquired

to conduct a double blind, placebo-controlled, antiparasitic treatment study (Garcia and others 2004). Patients ($n = 111$) were diagnosed as having at least one viable or inflamed cyst by CT or MRI imaging, and confirmed by EITB serology as outlined in Del Brutto (2001). All patients involved in the study were given antiepileptic drugs and were treated with either albendazole ($n = 56$) or placebo ($n = 55$) (Garcia and others 2004). Patients in both groups were monitored throughout the course of disease by both CT/MRI and EITB for periods ≤ 5 years.

For the purposes of the current study, the initial and final diagnostic serum samples collected from these patients were tested against three *T. solium* antigens to determine whether disease progression (shown by brain imaging) could be correlated with changes in seroreactivity. All patients who participated in this research provided written consent, and serum samples were collected in compliance with protocols approved by the ethical review boards of all institutions concerned (Johns Hopkins, CDC, Universidad Peruana Cayetano Heredia).

Taenia solium Antigens. The full-length, mature 8-kDa proteins, TS18var1 (AF098073) and TSRS1 (AF082830), were chemically synthesized by AnaSpec Inc., San Jose, CA at a purity of $\geq 95\%$. Both TS18var1 and TSRS1 were synthesized without their signal sequences, and are 67 and 66 amino acids long, respectively (Hancock and others 2003). The recombinant protein, GP50 (rGP50), is a glycosylated, GPI- anchored, membrane protein that was expressed in a baculovirus system. The predicted size of the mature protein portion of rGP50 is 28.9 kDa (Hancock and others 2004).

QuickELISA[™] Assay. The QuickELISA[™] (Marangoni and others 2005) cysticercosis assay kits for TS18var1, TSRS1, and rGP50 were developed and licensed by Immunetics, Inc., Cambridge, MA. All assays were conducted in 96-microwell plates according to manufacturer instructions. To perform the assays, we used the Trituris[®] EIA Analyzer (Diagnostic Grifols,

Barcelona, Spain), a programmable, automated, multi-batch enzyme immunoassay analyzer. The initial and final diagnostic serum samples for each patient were assayed side by side against each of the three *T. solium* antigens. Each assay plate included a 10-point standard reference curve with arbitrarily assigned unit values. Seroreactivity of individual patient samples was quantified based on the concentration values of standard curve units. Positive and negative control samples were included in each plate to ensure integrity of the assays. Positive control results that showed a coefficient of variance (CV) that was greater than 10% outside of the mean value were repeated. All patient samples were assayed twice, and sample results that showed a coefficient of variance (CV) that was greater than 10% between the two assays were repeated.

Analysis of QuickELISA™ Results. The QuickELISA™ assay data for each of the three antigens was evaluated separately. For each antigen assay, the changes in seroreactivity between the initial and final diagnostic samples were analyzed for each patient. Patients were categorized as having had a decrease in seroreactivity, or as having had either no change or an increase in seroreactivity by the conclusion of the study. Patients whose initial and final serum samples showed a mean value difference with a CV of less than 10% were considered to have no change in seroreactivity. Patients whose initial and final diagnostic samples showed no immunoreactivity against a particular antigen assay were excluded from analysis with that antigen.

Patient disease progression was measured according to changes in the number of viable cysts shown by CT/MRI between the point of initial diagnosis and final outcome. Disease progression was categorized in one of three ways: cured (having no cysts present or only calcified cysts at the end of the study), improved disease (having no viable cysts at the end of the study), or no change in disease status (having the same or an increased number of viable cysts at the end of the study).

Patients having only inflamed and single viable cysts were considered separately regarding assay sensitivity. Patient samples were evaluated as a unified study cohort, and further analyzed according to their respective clinical treatment protocols. Statistical relationships between observed and expected assay outcomes were analyzed using SAS® version 8 (SAS Institute, Inc., Cary, NC) software and SISA online statistical analysis (Uitenbroek 1997).

RESULTS

Albendazole Treatment versus Placebo. Statistical correlation of change in seroreactivity with disease progression was entirely independent of treatment protocol with all three antigens tested, showing a mean risk ratio of 0.95 ± 0.15 (CI, 95%). Because the individual clinical treatment protocols of these patients had no bearing on changes in seroreactivity in this study, patients were thereafter treated as one study group.

rGP50 QuickELISA™. This assay detected disease in 86 of the 111 (78%) patients tested. Sensitivity was low for patients showing only viable inflamed cysts (38%) and single viable cysts (85%). When patients with inflamed cysts were excluded, the sensitivity increased to 94%. By further excluding patients with only single viable cysts, sensitivity increased to 98%. Correlation between changes in rGP50 immunoreactivity and disease progression was significant ($P = 0.02$), as shown in Table 4.1. Statistical analysis of changes in the immunoreactivity of cured patients ($n = 44$) (who are a subset of those patients with improved disease) did not yield significant results ($P = 0.35$) (data not shown).

TS18var1 QuickELISA™. The overall sensitivity of this assay was 82% (91/111). Again, patients with inflamed viable cysts (66%), and single viable cysts (74%) showed low sensitivity.

Table 4.1. **Correlation of rGP50, TS18var1 and TSRS1 QuickELISA™ results with disease progression.** QuickELISA™ assay results from initial diagnostic serum samples drawn from neurocysticercosis patients were compared to assay results from serum samples drawn ≤ 5 years later. The change in seroreactivity between the initial and final serum samples was matched to corresponding disease progression as shown by patient CT/MRI. Pearson's chi square analysis was used to determine the significance of the correlation between seroreactivity change and disease progression for each antigen.

QuickELISA™ Antigen	Patient Disease Progression	Decreased Seroreactivity	Unchanged or Increased Seroreactivity
rGP50 P = 0.02	Improved (<i>n</i> = 54)	(26) 48%	(28) 52%
	No change (<i>n</i> = 32)	(7) 22%	(25) 78%
	Total (<i>n</i> = 86)	(33) 38%	(53) 62%
TS18var1 P = 0.01	Improved (<i>n</i> = 58)	(40) 69%	(18) 31%
	No change (<i>n</i> = 33)	(14) 42%	(19) 58%
	Total (<i>n</i> = 91)	(54) 59%	(37) 41%
TSRS1 P = 0.50	Improved (<i>n</i> = 68)	(35) 51%	(33) 49%
	No change (<i>n</i> = 36)	(16) 44%	(20) 56%
	Total (<i>n</i> = 104)	(51) 49%	(53) 51%

When patients with inflamed cysts were excluded, the sensitivity increased to 89%. Further exclusion of patients with single viable cysts increased the sensitivity to 96%. Correlation between change in TS18var1 seroreactivity and disease progression was significant ($P = 0.01$) (Table 4.1), whereas no significant positive correlation ($P = 0.18$) could be made between decreased seroreactivity and cured patients ($n = 47$) (data not shown).

TSRS1 QuickELISA™. Assay sensitivity was 94% (104/111). Sensitivity improved to 99% when inflamed cysts were excluded from this analysis. No significant correlation could be made between change in TSRS1 immunoreactivity and either improved disease ($P = 0.50$) (Table 4.1.) or cured disease ($P = 0.68$) (data not shown).

Seroconversion. Patients who had seroconverted from positive to negative within the time frame of the study were analyzed according to physiological cure (complete calcification or resolution of all cysts) as shown by CT/MRI imaging. Statistical analysis (two-sided Fisher's exact test) showed significant correlations between seroconversion and cured disease with both rGP50 and TS18var1 (Table 4.2). Six of the 44 (13.6%) cured patients tested with rGP50 QuickELISA™ seroconverted to negative ($P = 0.02$), while 46.8% (22/47) of cured patients seroconverted to negative against the TS18var1 QuickELISA™ ($P = < 0.01$). Seroconversion was not correlative with cured disease ($P = 0.14$) using TSRS1 QuickELISA™.

DISCUSSION

Although the overall sensitivity of both TS18var1 (82%) and rGP50 (78%) QuickELISA™ was low, exclusion of patients who had only inflamed cysts and single viable cysts increased the sensitivity of these assays to 96%, and 98%, respectively. This lack of sensitivity for serum from

Table 4.2. **Correlation of rGP50, TS18var1 and TSRS1 QuickELISA™ patient seroconversion with disease cure as shown by CT/MRI imaging.** QuickELISA™ assay results from initial diagnostic serum samples drawn from neurocysticercosis patients were compared to assay results from serum samples drawn ≤ 5 years later. Patients who had seroconverted from positive to negative were analyzed (two-sided Fisher's exact) according to their respective disease progression as shown by CT/MRI imaging.

QuickELISA™ Antigen	Patient Disease Progression	Seroconverted
rGP50 <i>P</i> = 0.02	Cured (<i>n</i> = 44)	(6) 14%
	Not cured (<i>n</i> = 42)	-----
	Total (<i>n</i> = 86)	(6) 7%
TS18var1 <i>P</i> = < 0.01	Cured (<i>n</i> = 47)	(22) 47%
	Not cured (<i>n</i> = 44)	(3) 7%
	Total (<i>n</i> = 91)	(25) 27 %
TSRS1 <i>P</i> = 0.09	Cured (<i>n</i> = 57)	(6) 11%
	Not cured (<i>n</i> = 47)	(1) 2%
	Total (<i>n</i> = 104)	(7) 7%

patients with light infections is not problematic, as it is common practice to determine cysticercosis assay sensitivity by analyzing serum from only those patients having multiple cysts (Scheel and others, in press). For the purposes of this study, insensitivity to serum from patients having only inflamed cysts increases the potential of these two antigens for monitoring patient disease progress. TSRS1 QuickELISA™ proved more sensitive to patients with inflamed and single viable cysts, and likely because of this, could not be shown to correlate with disease progression.

Although there have been previous studies that correlated quantitative *T. solium* antigen immunoreactivity with disease pathology (Machado and others 2002; Molinari and others 2002; Lopez and others 2004), no study has yet correlated quantitative immune response evolution with longitudinal disease progression within a neurocysticercosis patient cohort. All previous longitudinal studies have utilized the EITB (Garcia, Gilman, Catacora and others 1997; Meza-Lucas and others 2003) to monitor patient seroreactivity and examine changes in serorecognition of diagnostic antigen bands. An interesting observation resultant of these, and other studies (Ito and others 1999; Garcia and others 2001) is the frequent occurrence of seroconversion from positive to negative among persons exposed to *T. solium* infection. Large-scale field studies in endemic areas have shown that up to 40% of persons testing positive for exposure to *T. solium* infection spontaneously become seronegative to EITB antigens in less than one year (Garcia and others 2001). It has been speculated that this phenomenon may occur in either mild cases of disease that have resolved naturally, or in cases of exposure where infection has failed to establish. In this study, both rGP50 ($P = 0.015014$) and TS18var1 ($P = 0.000012$) QuickELISA™ (Table 4.2) have shown that seropositive patients with established infections do

become seronegative upon calcification of biologically active cysts. In fact 47% of TS18var1 QuickELISA™ seropositive patients later tested negative after physiological cure.

It has often been postulated that cysticercosis antigen detection assays alone will be useful for monitoring infection status of patients. To detect circulating *T. solium* antigen, ELISA assays have been developed using monoclonal antibodies raised against *Taenia saginata* antigens proven to elicit immune response in NCC patients (Harrison and others 1989; Brandt and others 1992). By detecting direct antigen products of biologically active cysts, these ELISAs clearly distinguish active from inactive cases of disease (Garcia, Parkhouse and others 2000; Fleury, Hernandez and others 2003). Although these ELISAs are valuable for establishing active infection, they are unable to distinguish between *T. saginata* and *T. solium* infections, negating their value in field studies because areas of endemnicity of *Taenia* parasites are known to overlap.

The cysticercosis QuickELISA™ assays evaluated here are the first evidence that immunoassays may be used as effective indicators of neurocysticercosis disease progression. Both TS18var1 and rGP50 QuickELISA™ show great potential with regard to improving our understanding of cysticercosis immune response dynamics, and with further development, may provide clinicians with a practical tool for patient management.

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CHAPTER 5

CONCLUSIONS

Implementation of control programs to reduce transmission of *Taenia solium* infections has catalyzed the need for a sensitive and specific diagnostic assay that is conducive to high-throughput applications. The EITB has been considered the “gold standard” immunoassay for detection and surveillance of cysticercosis in humans and pigs since its development in 1989 (Tsang and others 1989). Because of the unwieldy immunoblot format of this assay, as well as its reliance on native cyst antigens, use of the EITB for disease surveillance is difficult. In order to supersede the impractical aspects of the EITB, we reproduced its native glycoprotein antigens in synthetic and recombinant forms.

Four synthetic antigens, TS14, TS18var1, TSRS1 and TSRS2var1 (Greene and others 1999, 2000; Hancock and others 2003) were initially applied to the rapid FAST-ELISA format to determine their diagnostic potential for detection of cysticercosis. Each antigen candidate was directly adsorbed to polystyrene in order to capture the cysticercosis-specific antibodies of infected patients (Scheel and others, in press). Pilot tests showed that the pooled, positive serum standard reference curve we were using was immunologically biased. To circumvent this bias, a new standard curve was constructed using normal human IgG in order compare the seroreactivity of these antigens on a universal scale. We then evaluated the synthetic antigens for sensitivity and specificity against defined serum cohorts using our FAST-ELISA format. Results of these assays indicated that none of the synthetic antigens were highly specific for cysticercosis, and

one antigen, TSRS1, also showed very low sensitivity to infected patient sera (Scheel and others, in press).

The poor performance of these antigens in FAST-ELISA prompted the decision to assess their diagnostic efficacy in the EITB format in which they were originally defined.

Nitrocellulose test strips were probed with defined serum cohort samples to determine the sensitivity and specificity of each antigen. Two antigens, TS18var1 and TSRS1, performed well in the EITB format. TSRS1-EITB, in particular, was sensitive to patients with single viable cysts, and showed strong reactive bands when probed with sera from patients who had viable cysticercosis infections (Scheel and others, in press). The EITB assay format, however, is not conducive to the high-throughput testing that would be required to monitor disease in large geographical areas. An ELISA-formatted assay would be ideal for these purposes because it lends itself to automated processing, which is more accurate and expedient than manual pipetting of reagents.

In order to develop a sensitive, specific ELISA to detect cysticercosis, we partnered with a commercial immunodiagnostic laboratory, Immunetics, Inc., a licensee of the EITB. Immunetics, Inc. had recently developed successful ELISA assays for both Lyme disease and anthrax using a patented assay format, the QuickELISA[™] (Marangoni and others 2005). The QuickELISA[™] is a liquid-phase immunoassay that utilizes the strong non-covalent interactions between biotin and streptavidin (Chaiet and Wolf 1964) to adhere the analytes to the polystyrene matrix for detection. In the QuickELISA[™], neither antigen, nor antibodies have contact with the polystyrene plate. When the *T. solium* antigen candidates, TS18var1, TSRS1, and a recombinant *T. solium* antigen rGP50 (Hancock and others 2004), were applied to the QuickELISA[™] assay format and tested against clinically defined positive and negative serum cohorts, all performed

with much higher sensitivity and specificity than in the previous trials using the FAST-ELISA and EITB assay formats.

In the FAST-ELISA assay format, antigen is bound directly to the polystyrene. Synthetic antigens TS18var1 and TSRS1 (Scheel and others, in press), and recombinant antigen rGP50 (Bueno and others 2004) proved to be either insensitive or cross-reactive in the FAST-ELISA. In the EITB format, the protein antigens are linearized with sodium dodecyl sulfate (SDS). Both TSRS1 and TS18var1 showed much higher sensitivity and specificity to the defined serum cohorts in the EITB format. In the liquid-phase QuickELISA[™] format, antigens are not bound to polystyrene for antibody detection. All three of the antigens assayed in this format proved highly sensitive and specific. Clearly, the disparity in the FAST-ELISA, EITB, and QuickELISA[™] assay results indicate that antigen configuration and presentation are critical to assay performance.

The poor performance of the FAST-ELISA may be attributed to the binding of the antigen to the plate, resulting in a change in conformation that is unfavorable for recognition. Alternatively, the naked protein antigens may acquire a pseudo-conformation prior to binding the plate, resulting in recognition by antibodies previously unexposed to their *in situ* conformations. Interestingly, the linearized synthetic proteins of the EITB performed with much higher efficacy than FAST-ELISA, mirroring the sensitivity and specificity of their native counterparts in the LLGP-EITB. In the Quick-ELISA[™] format, both synthetic and recombinant *T. solium* antigens produced efficacious assays. Because the antigens are not bound to a matrix in this assay format, it is possible that their conformation is more similar to that of their native, *in situ* state, and thus, they are more sensitive and specific for cysticercosis-positive antibodies.

Because the *T. solium* antigen QuickELISA™ assays had proven highly efficacious for cysticercosis detection and had quantitative capabilities, we were prompted to explore the possibility of distinguishing biologically active cases of neurocysticercosis from residual cases of disease. We tested TS18var1, TSRS1 and rGP50 Quick-ELISA™ assays against a cohort of clinically-defined longitudinal sera to determine whether changes in seroreactivity correlated with disease status as shown by neuroimaging. Patients who showed improved disease status on neuroimages, meaning that the parasites they harbored had degenerated from viable, or biologically active states, to inactive states, showed a significant correlation with decreased seroreactivity to both TS18var1 and rGP50 QuickELISA™ assays. The TSRS1 QuickELISA™, however, showed no such correlation. Patients who were cured, or harbored only calcified cysts at the end of the study, showed significant correlation with TS18var1 and rGP50 QuickELISA™ seroconversion from positive to negative. Again, no such correlation could be made with the TSRS1 QuickELISA™. Concordant with this data, patients who had only single viable or inflamed cysts throughout the study were not always recognized by TS18var1 and rGP50 QuickELISA™. Conversely, TSRS1 QuickELISA™ showed little discrimination between viable and degenerating states of disease, resulting in higher sensitivity, overall. Although high sensitivity is ordinarily desirable in an assay, lower sensitivity to inactive cases of disease is critical for the determination of patient disease status.

The rationale for discrimination between active and inactive cases of NCC is based on recent findings that antiparasitic treatment with albendazole reduces the severity of symptoms during the inflammatory stages of cyst degeneration (Garcia and others 2004). Such discrimination between patients with active cases of disease, who may benefit from antiparasitic chemotherapy, and those with inactive cases of disease, in whom chemotherapy will be wasteful and

contraindicative, may prove beneficial to clinicians. The TS18var1 and rGP50 QuickELISA™ assays may be utilized as patient management tools when making decisions regarding antiparasitic treatment. Furthermore, for physicians in remote locations, such as Irian Jaya, New Guinea, the treatment protocol for NCC-positive patients is to provide antiparasitic medication for *all* who test positive for the disease, as no neuroimaging equipment is available (Sukwan Handali, personal communication). An assay that can discriminate between active and inactive cases of NCC would reduce expenditure on medication in areas with limited healthcare budgets. Discrimination between active and inactive cases of disease may also prove useful in field studies, as cysts degeneration is relative to the duration of an infection. Immunodetection of primarily active cases of NCC clustered within a given area could lead investigators to the human transmission source and possibly divulge information regarding the disease history and dynamics in that locale. Lastly, a quantitative immunological assay for cysticercosis may allow us to recognize patterns of humoral immune response to this disease.

The QuickELISA™ assays are prepackaged for ease of use in both clinical and field situations. All analytic reagents required to perform the assay are included, and quality control is performed by the manufacturer prior to packaging, lending assurance and reliability to user results. The simple format and quick development time of the QuickELISA™ is especially advantageous for field situations, as the results can be analyzed in roughly an hour. The assays results must be read by a spectrophotometer, however, so field stations maintaining such equipment would be necessary. In order to fully take advantage of the microwell plate format, an automated enzyme immunoassay (EIA) analyzer would provide for greater accuracy, as well as high-throughput analysis. The TS18var1 and rGP50 QuickELISA™ assays are currently being used at a field station in Tumbes, Peru to detect cysticercosis in both pigs and humans, and have

proved to be very robust for field use. The major disadvantage of the QuickELISA™ format is its inability to perform on-site testing. For these purposes, a lateral flow or dipstick assay would be required. We are currently developing such an assay with TS18var1, TSRS1 and rGP50 for use in extremely remote locations. The dipstick assay, however, will not allow for quantitation and disease status discrimination.

We are also developing three other *T. solium* antigens for eventual application to the QuickELISA™ format. A recombinant cysticercosis protein from a different LLGP antigen family, T24 (Kathy Hancock, unpublished data) is undergoing optimization for application to this format. Once optimization is complete, we will compare the efficacy of the T24 antigen to those of TS18var1 and rGP50 to determine its utility. Two recombinant proteins derived from adult tapeworms, the *T. solium* excretory/secretory antigens TSES33 and TSES38 (Levine and others 2004) are also undergoing optimization for application to QuickELISA™. Development of these serological assays will prove extremely useful in field situations, as no stool collection would be necessary to detect taeniasis carriers. The most efficacious TSES antigen assay will effectively replace the current coproantigen ELISA test. We are currently evaluating the TS18var1 and rGP50 QuickELISA™ assays with a greater number of clinically-defined serum in order to confirm which will be the most efficacious for NCC surveillance. Our eventual goal in developing QuickELISA™ assays using several cysticercosis antigens is to ascertain which antigen is the most efficacious, so that only one QuickELISA™ test is recommended for detecting infection in both humans and pigs. Currently, TS18var1 QuickELISA™ shows a strong correlation with active disease in humans. Additionally, the TS18var1 FAST-ELISA has shown that a dramatic drop in pig seroreactivity occurs after effectual treatment with oxfendazole (Handali and others 2004). TS18var1 QuickELISA™ will detect only those pigs with active

infections, whereas rGP50 QuickELISA™ may detect the passively transferred antibodies to GP50 that have been seen in the serum of piglets born to infected sows (Gonzalez and others 1999).

The field studies that are ongoing in Tumbes, Peru will be funded for a period of seven years by a US\$15 million grant provided by the Bill and Melinda Gates Foundation. The Tumbes project is the first comprehensive program designed to eliminate the diseases caused by *T. solium* in an endemic area. Currently, the Cysticercosis Working Group in Peru (CWGP) is monitoring both porcine and human disease using the QuickELISA™ immunoassays, as well as the coproantigen ELISA. In Tumbes, pigs are routinely treated with oxfendazole when serum is drawn, and humans found to carry tapeworms are given niclosomide to prevent further transmission. The CWGP is also collaborating with researchers worldwide in the development of a porcine vaccine against *T. solium*, as well as a monoclonal antibody for the detection of circulating *Taenia* antigen in human serum. The most important step in realizing control of *T. solium* in Tumbes, however, has been the development of the cysticercosis QuickELISA™ immunoassays.

The initiation of such a control program requires that several components be in place to ensure success. Suitable study areas must have a stable government with a well-structured, compliant health care administration. The area of study would also require the presence of an established cysticercosis research group of neurologists, veterinarians and physicians. Certainly, the assurance of long-term funding would be necessary when attempting to organize such a program. Surprisingly few endemic areas, however, meet the criteria above for successful surveillance and control of cysticercosis. Peru, Mexico, India, and Brazil are the most likely places to successfully administer limited control programs at this time. There are, however,

established research groups in Columbia, Tanzania, and Nepal, yet government instability is a constant threat to the progress of ongoing research in these countries. The long-term funding of future control programs for cysticercosis may well depend on the success of surveillance and control in Tumbes, Peru. Notwithstanding, all future surveillance and control programs for cysticercosis transmission will require a simple assay for detection of reservoirs of disease. The cysticercosis and taeniasis QuickELISA[™] assays are likely to prove critical for the movement toward potential eradication of diseases caused by *T. solium*.

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