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Neurocysticercosis

Diagnostics and vaccine development against the tapeworm, *Taenia solium*

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0. Summary

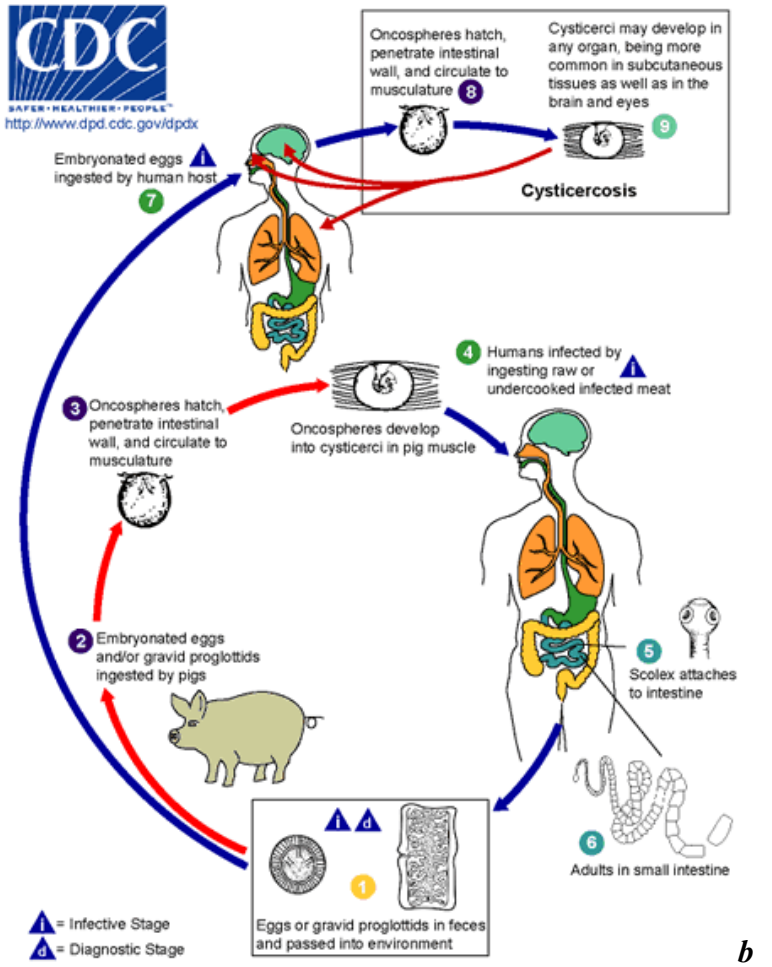
Taenia solium is a tapeworm infecting over 50 million people worldwide. It is particularly common in the developing countries of Africa, Latin America, and Asia, though reports of infection are increasingly noted in the United States and Europe. The tapeworm requires the pig as its intermediate host. When humans accidentally ingest its eggs in infected pork, contaminated vegetation, or by auto-infection induced by poor hygienic conditions, the severe and sometimes fatal disease neurocysticercosis may result. In this project, diagnostic methods for neurocysticercosis and vaccine development against *T. solium* were explored. It is shown that the Western blot immunoassay method is a useful tool for the diagnosis of neurocysticercosis in an epileptic population. Furthermore, results also support previous findings suggesting an association between neurocysticercosis and epilepsy. In the second part of this project, a construct for use in an edible vaccine was created using a novel *T. solium* cysticerci protein. The next steps of the novel edible vaccine development were simulated using two generations of the plant *Arabidopsis thaliana*, which had been previously transformed at Örebro University with the well-characterized *T. solium* protein, Tsol18. The first generation was examined using PCR analysis and showed that some of the plants were positive for the protein of interest. Protein analysis of the second generation was inconclusive but suggest that the Tsol18 protein may be present in the leaves of the plant.

1. Introduction

1.1. *Taenia solium*

1.1.1. Lifecycle

Taenia solium is a parasitic tapeworm whose life cycle requires the infection of pigs as intermediate hosts before infecting humans, the definitive hosts. Pigs acquire the infection through the consumption of vegetation contaminated by the parasite's eggs or gravid proglottids (body segments) themselves. The parasites then mature to oncospheres (embryos) in the pig's intestine, which can break the intestinal wall and develop into infective cysticerci (larvae) in the muscles. At this point, the infective cysticerci may be unknowingly ingested in undercooked pork by humans, migrate to the small intestine, and mature into adult tapeworms within two months. The adult tapeworms can survive in the intestine for years by attaching to the intestinal wall with their scolex (hooked structure, as shown in Figure 1a), resulting in the disease taeniasis. Hundreds of thousands of eggs can be released by the adult tapeworm during this time and relocated to the environment in pig feces or human excrement. When embryonated eggs themselves are ingested by humans, hatched oncospheres can circulate to the musculature, resulting in the disease cysticercosis. Neurocysticercosis occurs when the cysticerci circulate to the brain.



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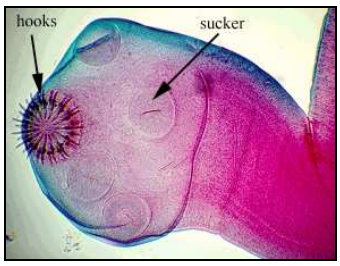

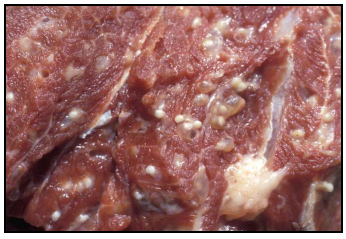

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Figure 1. *a.* (Top) Adult *T. solium* scolex with hooks and sucker labelled. Reprinted with permission from Professor Gary E. Kaiser (Maryland, USA). (Bottom) Pork meat infected with *T. solium* cysticerci. From the Armed Forces Institute for Pathology (Washington, D.C., USA).
b. Picture of the life cycle of *T. solium*. Steps 1-6 show the "typical" infection path, while steps 7-9 illustrate the path of autoinfection. From the Center of Disease Control (Georgia, USA).

1.1.2. Range and phylogenetics

Taenia solium is found nearly worldwide and is particularly prevalent in areas where small-scale pig farming or pigs as household livestock is common because it requires pigs as its intermediate host (García *et al.* 2006). Therefore, the developing countries of Latin America, Africa, and Asia are home to most instances of the tapeworm.

An increasing number of studies have evaluated the phylogenetic relationship among individuals of the *T. solium* species through molecular genetics and even historical methods and generally agree that there exist two sub-lineages of the tapeworm: One in Asia and one in Latin America and Africa (Nakao *et al.* 2002; Martinez-Hernandez *et al.* 2009; Hoberg 2005). From both a medical and clinical research perspective, this is important. Symptomatology may differ between the two lineages, thereby creating a need for different treatments or different treatment approaches depending on which area of the world the tapeworm originated. For example, it has been shown that the location of the cysticerci in afflicted Asian patients differs from that of Latin American or African patients and could contribute to differences in the clinical pictures of these patients (Nakao *et al.* 2002).

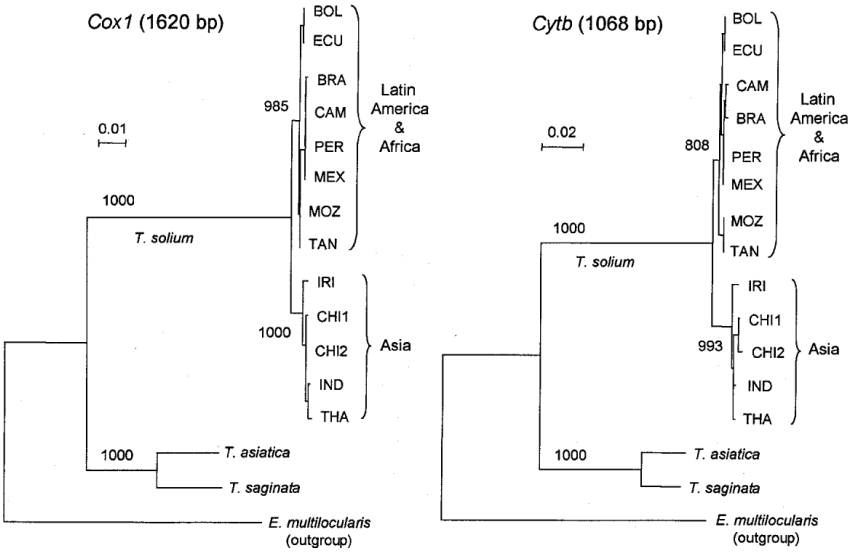


Figure 2. Phylogenetic evidence supporting two lineages of *T. solium*. Here, genetic sequence evidence from the *Cox1* and *Cytb* genes of cysticerci were compared. Both analyses separated cysticerci in Latin America (Mexico, Peru, Ecuador, Bolivia, Brazil) and Africa (Tanzania, Mozambique, and Cameroon) from those in Asia (China, Thailand, Irian Jaya, India). The neighbor-joining method was used (bootstrap values after 1000 runs shown). (Nakao *et al.* 2002.)

Another potential problem derived from the two *T. solium* lineages considers diagnostics and vaccine development. Many of the current methods for diagnosis of clinical infection involves the identification of specific antigens in patient sera; if antigen variations exist, it may prove impossible or impractical to try to create a universal diagnostics method. Similarly, many of the current approaches for vaccine development involve the creation of recombinant vaccines using immunogenic proteins; if variations of those antigens in terms of quantity, life stage, or otherwise exist between the two lineages, certain vaccines may be successful in one part of the world but less successful in another. Additional research about the clinical effects of the differences between the two *T. solium* lineages, while considering factors including nutritional differences and ethnic variation of the hosts, must be performed in order to elucidate a clearer clinical understanding of the potential impact the phylogeny of the tapeworm may have on humans and pigs alike (Nakao *et al.* 2002).

1.1.3. Disease

Human infection by *T. solium* causes two diseases: Taeniasis and cysticercosis. Taeniasis is marked by infection of the adult tapeworm in the intestines of humans and is most often acquired by eating poorly cooked infected pork. The disease is most often asymptomatic or presents mild, general symptoms such as nausea or abdominal pain. Although taeniasis is relatively harmless to its host, the danger of the disease is that it allows further proliferation of the tapeworm as its proglottids and eggs are expelled in host feces (García *et al.* 2003). The subsequent accidental ingestion of eggs by humans with close contact with the host or even by the host themselves can lead to more dangerous *T. solium* infections in other humans, namely cysticercosis and neurocysticercosis.

Cysticercosis (CC) and neurocysticercosis (NCC) are diseases characterized by cyst development in the eyes, skeletal muscle, heart, or brain caused by the ingestion of *Taenia solium* eggs. NCC is particularly devastating and is the focus of most research efforts targeting the tapeworm. Symptoms can range from the clinically mild to the clinically severe, in which epileptic seizures, hydrocephalus, or localized neural deficits precede *T. solium*-associated fatalities. Over 20 million people worldwide are estimated to be infected with either the adult tapeworm or its larvae and its wide-ranging medical, social, and economic effects are felt globally (Pawlowski *et al.* 2005).

1.2. Cysticercosis and Neurocysticercosis

1.2.1. Pathology and symptoms

NCC is one of the leading causes of epilepsy in the developing countries of Asia, Latin America, and Africa. The disease develops when *T. solium* oncospheres emerge from their eggs, enter the bloodstream, and embed themselves in the brain, where they develop into cysts. The cysts often, but not always, stimulate an immune response, particularly when undergoing degradation but also when first active and following calcification; it is this immune response that leads to the disease's symptoms, including epileptic seizures (Nash *et al.* 2004).

Over fifty million people worldwide suffer from epilepsy, three quarters of which live in developing countries. Previous studies have shown that NCC is a significant risk factor for epilepsy in both Latin America and Africa (Garcia *et al.* 1999; Pal *et al.* 2000; Nsengiyumva *et al.* 2003; Preux and Druet Cabanac 2005). Rigorous studies showing an association between epilepsy and neurocysticercosis in areas of Asia are less common in the literature. However, there are studies that show that up to fifty percent or even eighty percent of CC patients in India and Nepal have had seizures, and similarly that between eleven and fourteen percent of epileptic patients in Bali also tested positive for *Taenia solium* infection (Theis *et al.* 1994; Rajshekhar *et al.* 2003). It is important to note that a clinical distinction between epilepsy and seizures does exist, and that seizures may occur without patients being medically diagnosed as epileptic.

1.2.2. Range and prevalence

Different forms of the disease occur depending on where infection, including autoinfection, by the fecal-oral pathway occurs. Furthermore, infected humans can transmit new *T. solium* eggs or gravid proglottids in their stool, which allows the cycle to be repeated when pigs consume newly contaminated vegetation. Eating infected pork or raw, contaminated vegetables is the most common path of ingestion in the developing countries of Asia, Latin America, and Africa where the diseases are most prevalent. However, due to immigration and

increased movement of people across country borders, CC and NCC are becoming increasingly prevalent in developed countries.

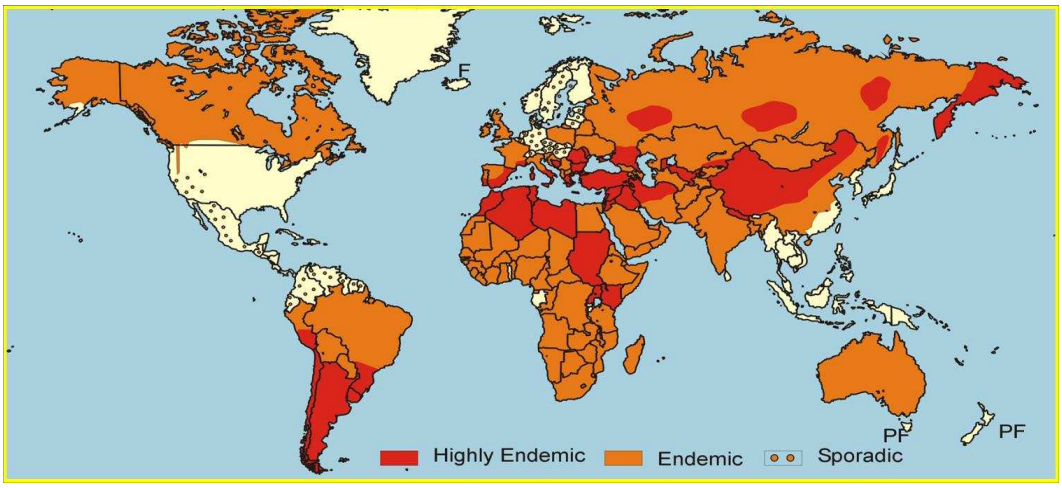


Figure 3. Map of areas affected by neurocysticercosis and cysticercosis. From the World Health Organization (Geneva, Switzerland).

The United States is one country reporting an increasing number of cases of the imported *T. solium* diseases; in Los Angeles County, California, studies in the 1980s repored an average of 48 cases of NCC cases admitted to the hospital every year, while in the 1990s that number jumped to 94 cases per year (DeGiorgio *et al.* 2002; Scharf 1988). Furthermore, according to a study published in 1994, NCC is increasing by approximately one thousand new cases each year in Houston, Texas and the phenomenon is not only limited to the southwestern United States: cases of NCC have been reported in children in Chicago as well as in members of an Orthodox Jewish community in New York City (Shandera *et al.* 1994; Rosenfeld *et al.* 1996; Schantz *et al.* 1992). The primary populations in which the studies focus are migrant farmers and the Hispanic community because migration from endemic areas, such as Latin America, is widely thought to be an important cause of the increase in NCC incidence in the United States. Other causes include travel to endemic areas or close contact (such as sharing a household) with often unknowingly infected individuals (Mitchell *et al.* 2004). Despite local monitoring efforts that attempt to capture a picture of NCC incidence in countries such as the United States, its range of symptoms make this a challenging task. The 2004 study by Sorvillo *et al.* attempted to determine the number of CC-related deaths in California over a 12-year period. The authors observed that, in their opinion, a more accurate account of actual CC deaths could be determined by including multiple causes of death data in their research; 32 of their total 124 cases of death (26%) were attributed to symptoms of CC rather than the disease directly, including combinations of hydrocephalus and epilepsy or convulsions (Sorvillo *et al.* 2004).

1.2.3. Social and economic effects

Both CC and NCC present heavy public health and economic burdens for developing countries and are increasing problems for affluent countries unknowingly accepting immigrants infected with the tapeworm. Socially, associated symptoms of NCC such as epilepsy cast afflicted residents of small communities to the fringe of society; one study noted that less than 30% of epileptics marry and less than 40% become professionally employed in West Cameroon (Zoli *et al.* 2003). Furthermore, the economic losses caused by the death of infected pigs are staggering considering the limited economic resources of the areas most

afflicted by the parasite (Flisser 1988; Murrell 1991). In one review examining the cost of foodborne parasitic diseases, the human health costs for one case of NCC in 1994 in the United States totaled US$8 055, including hospitalization costs and lost wages (Carabin *et al.* 2005). In the same decade, treatment costs in Brazil reached into US$85 million, without considering the costs of other illness associated with NCC, such as epilepsy (Roberts *et al.* 1994).

Economic losses due to NCC are not limited to human health or lost income costs. The financial loss due to infected pigs in Mexico alone was estimated at over US$43 million, or over 50% the national investment in pig production (Carabin *et al.* 2005). Similar numbers are reported across Latin America as well as in Africa, and the people hit hardest across the world are the small-scale pig farmers (Tsang and Wilson 1995). Thus, the use of an edible vaccine targeted to pigs could provide the safest, most cost-effective pathway to combat *T. solium* infection in pigs and humans alike (Molinari *et al.* 1997; Huerta *et al.* 2001). Researchers working in Latin America, Asia, and Africa suggest that, if combined with public health education about *T. solium* transmission, the vaccination of pigs in endemic populations could lead to the eventual eradication of CC in both porcine and human populations, with tremendous savings on both social and economic levels (Sciutto 2000; Sotelo 2003; Gonzalez 2005).

1.2.4. Diagnosis

Limited diagnostic capabilities limit the treatment of CC and NCC. Imaging technologies such as computed tomography (CT) and magnetic resonance imaging (MRI) show the greatest diagnostic success, with limited false positives and false negatives (White 2000). However, such technologies are both technical and expensive and therefore often inaccessible to the developing countries in which the diseases have the greatest effect. Furthermore, CT and MRI are effective in identifying cysticerci that have already migrated to the brain and caused NCC; a diagnostic method aimed at earlier identification of the disease would allow for more effective treatment.

Enzyme-linked immunosorbent assay (ELISA) or enzyme-linked immunotransfer blot (EITB) are other diagnostic tools and work by detecting the presence of antibodies for specific (or recombinant) cysticerci-associated antigens found in sera. Although these tools can show relatively high sensitivity and are more accessible in terms of cost and use than the aforementioned imaging tools, major limitations such as low specificity, poor results in patients with single or calcified cysts, and moderate implementation difficulties in developing countries keep these methods still less than ideal (García *et al.* 1998; Gekeler *et al.* 2002; Castillo 2004). These tests are particularly unreliable when the patient is infected with other parasitic flatworms such as species from *Schistosoma* or *Echinococcus* genera, as cross-reactions are known to occur (Diwan *et al.* 1982; Pammenter *et al.* 1992). However, methods for purifying cysticerci to improve results with EITB do exist. Important diagnostic glycoproteins in crude homogenized cysticerci that are passed through a lentil lectin affinity column, for example, bind to the column and can be eluted later to yield a “cleaner” extract; in an early study by Rodriguez-Canal *et al*., lectin-purified antigens yielded 95% sensitivity as compared to 91% sensitivity with crude antigens (Rodriguez-Canal *et al.* 1997). In the field, a seroprevalence of 9.6% was detected in a randomly selected population from Bali when using purified extract as compared to 7.8% with crude extract (Rodriguez-Canal *et al.* 1997). These findings suggest that purified extracts provide more sensitive and therefore more conclusive diagnostic results.

1.2.5. Treatment

Medications used to combat *T. solium* infection in humans are the vermicides albendazole and praziquantel. Treatment with both drugs is controversial, however, since studies have shown mixed efficacy results particularly when treatment is combined with other medications (such as antimalarial therapies) and side effects may include increased inflammation, which can amplify already severe symptoms (Sciutto *et al.* 2000; García *et al.* 2003; García *et al.* 2004; Carpio *et al.* 2008). Much treatment is also geared towards controlling the symptoms, such as anti-epileptic therapy or therapy aimed at managing intracranial hypertension or oedema (García *et al.* 2003). It is widely recognized that a proactive approach to *T. solium* infection (such as vaccination) may provide the best form of treatment by rendering treatment unnecessary.

1.2.6. Vaccinations, measures of control

Early studies researching the potential for vaccination as a measure of control of taeniid species found that compared to other helminth infections, transmission regulation is largely affected by immunity to re-infection, and that immune responses target the oncosphere (Lightowlers 2003). Furthermore, recombinant vaccines against *Taenia ovis* (sheep) and *Taenia saginata* (cattle) have been developed using immunogenic oncospheric antigens and not only are effective measures of CC control in both species but offer important theoretical and practical guidance for the creation of a *T. solium* CC vaccine (Lightowlers 2003).

No vaccine against *T. solium* infection is in use in humans, although the use of the antigen Tsol18 to create a vaccine for pigs shows promise (Cai *et al.* 2007). Other vaccine targets include the oncosphere antigen Tsol45, the recombinant antigen KETc7, and the peptides KETc1, KETc12, and GK1 (Manoutcharian *et al.* 2004; Gonzalez *et al.* 2005). Researchers and public health officials also name other potential, human-focused measures of *T. solium* control including blanket treatment with taenicidals or chemotherapy of infected individuals (Lightowlers 1999). Although these ideas may seem theoretically possible methods to eradicate taeniasis and CC, numerous obstacles to their success exist when they are considered realistically. For example, the cost and practicalities for a global campaign to treat everyone with anti-*Taenia* medication would be enormous and would require complete and universal compliance in order to be effective. Similar hurdles would face an attempt to provide chemotherapy to the over 20 million people who are thought to be infected with the tapeworm, or to design and hold clinical trials for vaccine use in humans. Furthermore, even if it was globally decided to allocate the resources to perform such treatments, the main source of *T. solium* – infected pigs – still would be host to the parasite, which can release over a quarter of a million eggs per day per tapeworm (Lightowlers 1999),

Therefore, for both practical and economic reasons, a research focus on porcine vaccines rather than human vaccines currently dominates the field. Studies focusing on the intramuscular administration of recombinant oncospheric proteins such as Tsol18, Tsol45-1A, and Tsol45W-4B have showed levels of protection between 74% and 100% and have significantly decreased the total number of cysticerci in experimentally infected pigs (Plancarte *et al.* 1999; Flisser *et al.* 2004; Luo *et al.* 2009). However, these vaccines require trained personnel, are relatively expensive to produce, and must be transported in relatively demanding conditions (such as with refrigeration). The current challenge for CC vaccine development is the creation of one which can be implemented effectively in field conditions; an edible vaccine is thought to provide the solution to this problem.

1.3. Edible Vaccines

1.3.1. Theoretical background

Recent work by Ingrid Lindh has shown that the production of edible vaccines can be successful using recombinant plants, and may even be a preferable method of administration for CC or NCC vaccines because like the parasite, an orally administered vaccine can stimulate a strong mucosal immune response (Thanavala *et al.* 2005; Lindh *et al.* 2008). Furthermore, vaccines administered by injection, puncture, or intranasally may be largely impractical in the localities most affected by *Taenia solium* infection. This is attributed to the large cost of transport, demanding storage and quality control requirements (such as refrigeration), potential health concerns including increased chance of blood-borne disease transmission to the vaccine provider, and difficulty with patient follow-up in the case of multidose regimens (Hopkins 1985; Clemens *et al*. 1996; Giudice and Campbell 2006). In contrast, a plant vaccine provides a low-maintenance alternative that is inexpensive, heat-stable, easily transported, and convenient (Streatfield *et al*. 2001; Streatfield and Howard 2003). Previous studies have shown that transgenic lupin, lettuce, and potatoes can provoke immune responses specific to the targeted virus or bacteria; in these cases, specific immune responses to both the hepatitis B virus and a synthetic peptide corresponding to enterotoxigenic *E. coli* were observed after both mouse and human volunteers consumed the transgenic plants (Tacket *et al.* 1998; Kapusta *et al.* 1999).

One of the common criticisms with plants as edible vaccines is that their dosage cannot be

research purposes support its unique position as a model for plant molecular biology (Meinke *et al.* 1998).

1.3.3. Example: *Feeding of mice with* Arabidopsis thaliana *expressing the HIV-1 subtype C p24 antigen gives rise to systemic immune responses*

A 2008 study at Örebro University, Sweden, recognized the benefits of a transgenic plant vaccine to combat HIV infections in sub-Saharan Africa, where the HIV-1 subtype is commonly found and where the need for an affordable and effective solution is critical (Lindh *et al.* 2008). In the study, researchers used the plant bacteria, *Agrobacterium tumefaciens*, to mediate gene transfer to *A. thaliana* of a specifically constructed vector containing the gene for the entire p24 *gag* antigen (specific to HIV-1) as well as selection markers for herbicide and antibiotic resistances. The seeds from the transformed *A. thaliana* plants were removed and grown on plates containing the herbicide BASTA, to which plants containing the vector should be resistant. All plants that grew on the plates were transferred to soil and grown until seeds could again be harvested; the process was repeated once more. PCR amplification on extracted DNA from the resulting *A. thaliana* plants showed the presence of the p24 *gag* gene. Further analysis to check for the p24 *gag* protein was completed using SDS-Page and Western Blot, with homogenized leaves, stems, and roots of the mature plants. Enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of p24 *gag* protein in the leaves and stems of the plants as well.

The effect of the transgenic *A. thaliana* on mice was determined via a two-part experiment. In the first part, five mice were given equivalent access to 10-12 grams of the fresh transgenic plant for 48 hours on day 0 and day 14 of the study, with their normal food. Blood was removed from the mice on day 0 and day 28 and examined for the presence of specific antibodies using ELISA. In the second, four groups of six mice each were given either 10-12 grams of fresh transgenic *A. thaliana*, freeze-dried transgenic *A. thaliana* (final weight 0.8-1 grams), fresh non-transgenic *A. thaliana*, or freeze-dried non-transgenic *A. thaliana* (final weight 0.8-1 grams). The freeze-dried plants were mixed with water to make a paste. Each group was given 48-hour access to their respective *A. thaliana* in combination with their normal food on days 0, 14, 35, and 70. Blood was removed from each mouse on days 28, 49, 84, and 189. On day 175, half of each group (excluding the control group, which ate fresh *A. thaliana*) was intramuscularly injected with either plasmid or recombinant p37 *gag* protein.

Results of this study showed that *A. thaliana*, transgenic for a specific HIV-1 antigen, can be created so that both the gene and the protein are experimentally observable and stable in subsequent generations. In this example, the p24 *gag* protein was present in highest amounts in stems and leaves but not at all in roots. It was also shown that when transgenic *A. thaliana* was consumed by mice that were later challenged with a protein designed to elicit an immune response, all mice fed with the fresh plant showed an immune response while only one-third of the mice given access to freeze-dried transgenic *A. thaliana* showed a response. Although this study represents the beginning of a specific example of edible vaccine development, the results provide “proof-of-principle” and suggest that with additional research, this novel approach could prove effective as one part of the fight against HIV infection (Lindh *et al.* 2008).

1.4. Project Aims

The general aim of this project was to investigate diagnostic methods and vaccine development in *T. solium* infection. Specific aims for this thesis were:

- To investigate any association between epilepsy and NCC in patients from Vietnam while testing the effectiveness of the Western blot immunoassay method in the diagnosis of NCC as compared to CT results
- To prepare a new edible vaccine using a novel protein from *T. solium* cysticerci
- To test two generations of transformed *A. thaliana* for the presence of a previously prepared vaccine vector containing a highly immunogenic protein from *T. solium* cysticerci

2. Project I: Diagnostics

2.1. Aim

This aim of this study was two-fold. First, the Western blot immunoassay method was used to determine its effectiveness as a tool for the diagnosis of NCC in comparison with the CT brain imaging method. Furthermore, the results of both analyses were used to investigate the association between NCC and epilepsy in a Vietnamese patient population.

2.2. Materials and Methods

2.2.1. Collection and preparation of samples

Collection of human sera. Sera from 28 epileptic patients in Hanoi, Vietnam, provided by Nguyen Anh Tuan (Department of Neurology, Hanoi Medical University, Hanoi, Vietnam) was used.

2.2. Preparation of antigens. Five cysticerci removed from a *T. solium*-infected pig in Nicaragua were homogenized in 200 µl PBS (Medicago AB PBS tablets, Sweden) and 1 µl protease inhibitor (Sigma Aldrich, USA) in a 2 ml Eppendorf tube. The tube was centrifuged at 1000 x *g* for 10 minutes, the supernatant containing the antigens transferred into a new tube, and the pellet discarded.

2.2.2. Western Blot

SDS-polyacrylamide gels. Ten percent SDS-PAGE solution gels[1] were prepared. Equal quantities of 1x SDS loading buffer[2] and antigen sample were mixed and heated at 96°C for 5 minutes. Ten microliters of the sample-buffer solution were added into each lane of the gel. The gel was run on Bio-Rad PowerPac™ Basic (Bio-Rad Laboratories Limited, United Kingdom) at 130V for 90 minutes in SDS-PAGE running buffer[3].

Western blot. Protein transfer from the gels to a membrane was performed using standard semi-dry electrophoretic transfer protocol. Three sheets absorbent filter paper were cut to match the size of the gels and dipped in semi-dry transfer buffer[4] and placed on the semi-dry apparatus (NovaBlot with Electrophoresis Power Supply EPS-3500XL; Amersham Pharmacia Biotech, United Kingdom). One sheet of Hybond-P membrane (Amersham Pharmacia Biotech, United Kingdom) also cut to match the size of the gel was carefully submerged in 100% methanol for 10 seconds and distilled water for several minutes and then placed on top of the filter paper. The gel was placed on top of the nitrocellulose membrane, the lanes carefully marked in pencil, and three additional pieces of filter paper (also soaked in semi-dry transfer buffer) placed on top. The apparatus was set to run at 45 mA (as limiting factor) for one hour.

Blocking was performed in Blotto blocking buffer[5] for one hour at room temperature on an agitator (IKA® KS 130 Basic, IKA®-Werke GMBH & Co.Kg, Germany). Separate lanes were cut from the membrane and placed in separate Petri dishes containing 10 ml 5% nonfat milk (5 g nonfat milk in 100 ml PBS (Medicago AB PBS tablets, Uppsala, Sweden)) with 20 µl human sera each. One CC-positive sample from Nicaragua and one CC-negative human

[1] See Appendix 7.1 and 7.2.
[2] See Appendix 7.6.
[3] See Appendix 7.3.
[4] See Appendix 7.4.
[5] See Appendix 7.5.

sera sample from Nicaragua also were used (10 ml 5% nonfat milk with 2 µl corresponding sera per Petri dish). The negative control remained in Blotto blocking buffer. All samples were agitated at room temperature for 90 minutes or overnight at 4°C.

All samples were washed three times with PBS + 0.05% PlusOne Triton X-100 (GE Healthcare, Uppsala, Sweden) solution for twenty minutes per wash. All samples were agitated during washing.

Ten milliliters of secondary antibodies (anti-human Immunoglobulin horseradish peroxidase-linked whole antibody (from sheep); Amersham Biosciences, United Kingdom) in 10 ml 5% nonfat milk were added to each sample following washing. The samples were agitated in separate Petri dishes at room temperature. After 60 minutes, the samples were washed three times for twenty minutes as performed previously.

2.2.3. Detection

Chemiluminescence detection was performed through development of film exposed to the washed nitrocellulose membrane. Standard protocol for the Amersham™ ECL Plus Western Blotting System (GE Healthcare, United Kingdom) was followed: One milliliter Solution A (Lumigen™ PS-3 detection reagent) and 25 µl Solution B (Lumigen™ PS-3 detection reagent) were mixed in a 1.5 ml tube. The nitrocellulose membrane was placed on the cassette and washed with the Solution A/Solution B mix so that the entire membrane was covered; excess solution mix was removed with a dry cloth. A clear film was placed over the soaked membrane and once in the dark room, the Amersham Hyperfilm™ ECL developing film (GE Healthcare Limited, United Kingdom) was exposed to the membranes for three minutes and standard developing protocol using the Curix 60 (Agfa Healthcare, Belgium) was followed.

2.2.4. Analysis

Gel-band patterns from the CC-positive serum were compared to gel-band patterns from the unknown samples. Approximate size, strength, and clarity were considered.

2.3. Results

Figure 4 shows the positive and negative controls for the Western blot immunoassay. Nicaraguan sera from patients who were either positive or negative for NCC infection (determined prior to this study) were used at a concentration of 1:5000 in the Western blot immunoassay. Band patterns from the NCC-positive Nicaraguan patient sera showed bands at 20 kDa and 50 kDa, while no bands are observed from the NCC-negative Nicaraguan patient sera. This indicates that no band patterns from the sample patient sera would suggest no NCC infection, while a band pattern (particularly at sizes 20 kDa and 50 kDa) would suggest NCC infection.

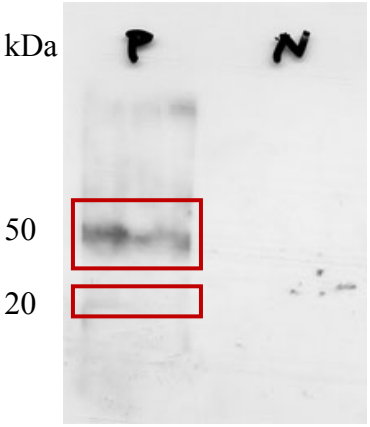


Figure 4. Western blot immunoassay on Nicaraguan sera. Positive (P) and negative (N) sera from Nicaraguan patients showing antigen band patterns for comparison to Western Blot immunoassays of Vietnamese patients. As expected, P showed bands at about 20 kDa and 50 kDa (in red) and N showed no bands.

Results from the Western blot immunoassay of all Vietnamese epileptic patient sera are shown in Figure 5. All patient sera that were considered NCC-positive by this method are arranged in Figure 6, next to 4 select NCC-negative patient sera for comparison. Of the 28 Vietnamese patients, 5 were considered NCC-positive by Western blot immunoassay. The band patterns displayed on the suspected NCC-positive patients shown in Figure 6 indicate proteins of molecular weights higher or lower than expected according to results of the NCC-positive control sample using Nicaraguan sera. This indicates that there may be a difference in the sera of NCC-positive Vietnamese epileptic patients and the sera of the NCC-positive Nicaraguan patient.

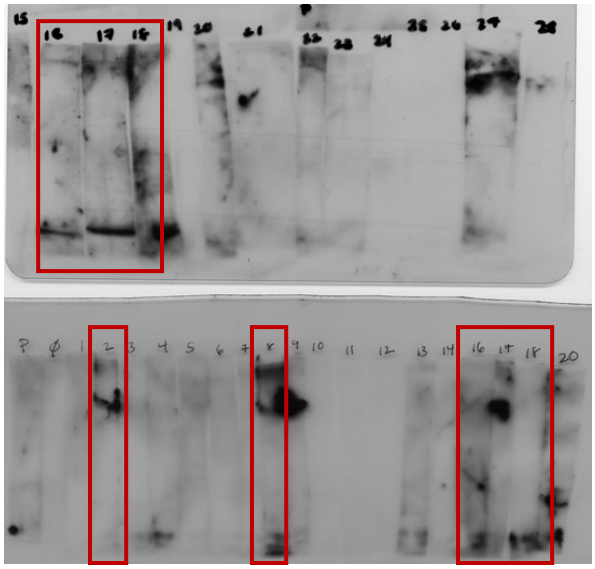


Figure 5. Western blot immunoassay of all Vietnamese epileptic patient sera samples. As is apparent by the number labels, some samples were immunoassayed twice. Strong positives are marked in red.

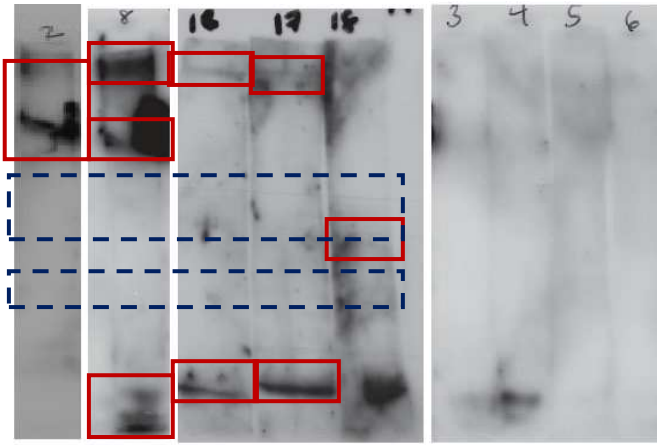


Figure 6. Western blot immunoassay NCC-positive sera samples compared to select Western blot immunoassay NCC-negative sera samples. The 5 samples on the left are the samples considered to be positive by Western blot immunoassay; samples 2 and 8 (far left) are those that were found to be positive by CT scan. Red boxes mark the bands while the blue dashed boxes mark the approximate locations of the bands of the positive control. The 4 samples on the right are negative samples and are provided as points of comparison to the positive

Of the 5 sera samples from Vietnamese epileptic patients that were considered NCC-positive by Western blot immunoassay, 2 patients were considered NCC-positive using the CT brain imaging method. These samples are labeled “2” and “8” in Figure 6 and show the most pronounced bands. Table 1 shows the percent sensitivity, percent specificity, and prevalence by both Western blot immunoassay and CT imaging. The results of the CT method were accepted as “true.” The findings displayed in Table 1 show that the prevalence of NCC in a Vietnamese epileptic patient population ranges between 7% (CT method) to 17% (Western blot immunoassay method). These numbers support previous studies exploring the association between NCC and epilepsy. Furthermore, the ability of the Western blot immunoassay method to detect NCC-positive patients (percent sensitivity) was 100% and to detect NCC-negative patients (percent specificity) was 89.66%. This suggests that the Western blot immunoassay method is a useful diagnostics tool for detecting NCC in an epileptic population but that some patients considered to be NCC-positive are actually NCC-negative, according to CT imaging.

Table 1. Calculation of various measurements of success of the Western Blot immunoassay method as compared to the CT brain imaging method. Each corresponding equation is also provided in the table.

Calculation	Percent (%)	Equation
Percent sensitivity	100%	$100\left[\frac{\text{True Positives}}{(\text{True Positives} + \text{False Negatives})}\right]$
Percent specificity	89.66%	$100\left[\frac{\text{True Negatives}}{(\text{True Negatives} + \text{False Positives})}\right]$
Prevalence (in epileptic patients, by Western blot immunoassay)	17.86%	$100\left[\frac{\text{Number of Western Blot Immunoassay Positives}}{\text{Total Number of Samples}}\right]$
Prevalence (in epileptic patients, by CT imagine)	7.14%	$100\left[\frac{\text{Number of CT Imaging Positives}}{\text{Total Number of Samples}}\right]$
True positives = 5 True negatives = 26		False positives = 3 False negatives = 0

2.4. Discussion

The aim of this study was two-fold: One, to determine the usefulness of the Western blot immunoassay method in the diagnosis of NCC as compared to previous results obtained from CT imaging and two, to compare the prevalence of NCC in this population of NCC patients with existing literature to help clarify the relationship between NCC and epilepsy in Asia. In short, results suggest that the Western blot immunoassay method is a useful diagnostics tool and that this small population supports previous findings about the prevalence of NCC in Asian epileptic patients.

Analysis of the Western blot immunoassay results revealed five potential NCC-positives, two of which were confirmed with CT imaging. No samples that were NCC-positive by CT imaging were NCC-negative by Western Blot immunoassay (100% sensitivity) but three samples considered to be NCC-positive by Western blot immunoassay were NCC-negative by CT imaging (89.66% specificity). This suggests that the Western blot immunoassay method is a useful and reliable method for the diagnosis of NCC in epileptic patients, but may overestimate or over-diagnose the number of patients actually inflicted with the *T. solium*-associated disease. Several factors could contribute to the number of false positives detected. First, crude antigen extracts were used. This provides an opportunity for cross-reactions to occur and present the potential positive results indicated by analysis. Therefore, there is a need to evaluate the crude extract and determine if purification of the sonicated cysticerci is necessary.

Another potential factor is that these results were based on an experimental design in which Vietnamese sera was tested on Nicaraguan cysticerci; known phylogenetic relationships based on analysis of *T. solium* mitochondrial and nuclear sequences (including but not limited to

co1, *cob*, *nad*, *LMWA1*, the 45W gene family, and *Tsol18*) have presented two lineages of the tapeworm, with one of Asian *T. solium* and the other with Latin American and African *T. solium*. This study mixes the two lineages by testing Latin American cysticerci with Asian sera samples. This could explain the discrepancies in band location observed in Figure 3 among the positive control, which used both Nicaraguan sera and Nicaraguan cysticerci, and the other "NCC-positive" samples, which used Asian sera and Nicaraguan cysticerci. There is no band among the sera samples that corresponds with those observed in the positive control; rather, the observed bands suggest proteins of either higher molecular weight or lower molecular weight than expected, perhaps due to unspecific binding or other worm infections. That the band pattern differs between the positive control and some of the samples is also true for sample 2, the other CT-confirmed NCC-positive, which shows no bands in the expected regions. Instead, for both samples 2 and 8, bands of approximately 80-100 kDa are observed. This could suggest the existence of an antibody specific to Asian *T. solium*, and merits additional study with more patient samples to determine whether the antibody denotes a novel *T. solium* antigen, whether (and why) it is location-specific, whether it could be a focus in vaccine development, or whether it could be used in the diagnostics of NCC in an Asian population. Furthermore, if the antibody is specific to Asian NCC, it could also help elucidate the evolutionary differences between Latin American and African *T. solium* and Asian *T. solium* and therefore deserves additional attention from both an infectious biology and evolutionary biology approach.

Further research within the realm of this current study that could clarify the role of evolution on diagnostic proteins of the two *T. solium* lineages, more precisely determine the success of the Western blot immunoassay method as a diagnostic tool, and contribute to the ongoing conversation about the importance of the two *T. solium* lineages regarding both diagnostics and vaccine development is to repeat the experiment using Asian cysticerci. That is, if a laboratory difference is observed between the results using Latin American cysticerci and Asian cysticerci, more location-specific attention could be recommended in both diagnostics and vaccine development. This would be particularly important to study for popular proteins such as Tsol18 (Gauci *et al.* showed that Tsol18 is highly, but not perfectly, conserved but did not test this under laboratory conditions, 2006). Furthermore, testing the provided Asian sera with Asian cysticerci could also reveal potential problems in diagnosis and vaccine development, such as the effect of other worm infections, specific to parts of Asia and excluding Africa and Latin America, on the use of Western blot immunoassay as a diagnostics tool. In fact, since little is known about which band patterns should be expected in Asian CC infection, it may be that the patients presumed to be NCC-positive by Western Blot immunoassay but found NCC-negative by CT brain imaging were indeed infected with a worm, but with one other than *T. solium*. The unexpected band pattern observed in sample 2 may be a patient with a currently active infection from another worm but an inactive *T. solium* infection, which may or may not be linked to their epilepsy.

The distinction between active and inactive worm infections is one that must be addressed in any discussion about the efficacy of the Western blot immunoassay method. The technique works by detecting antibodies in patient sera, which bind to antigens from homogenized cysticerci. Therefore, the method can only detect infections in which antibodies continue to circulate in the bloodstream. Little is written in the literature about the longevity of antibodies during CC infection, and it may be that the Western blot immunoassay method can only detect active worm infections and will neglect calcified cysts if antibodies to the cysts no longer exist in the patient. However, one recent study by Rodriguez *et al.* in Peru looked at the proportion of NCC-positive results of two different methods and specimen types for intraparenchymal NCC patients with viable cysts as well as patients with calcified cysts only

(Rodriguez *et al.* 2009). Under similar protocol to that used in this study, 100% (41/41) of the patients with viable cysts and 100% (7/7) of the patients with calcified cysts only were correctly identified as NCC-positive using sera. Similar but older ELISA-based studies testing sera, feces, or cerebrospinal fluid for the presence of *T. solium* antibodies in both patient and animal model populations also have shown that the presence of antibodies persists even with only calcified cysts (Corona *et al.* 1986; Zini *et al.* 1990; Molinari *et al.* 2002). This information further supports the usefulness of Western blot immunoassay as a diagnostic tool (at least in Latin America), but may also present difficulties regarding treatment. Treatment with antiparasitic medication only works against viable cysts and this method does not distinguish between active and inactive cysts.

In regards to the relationship between *T. solium* infection and epilepsy, results from this study show that 7.1% (by CT imaging diagnosis) of patients from this small epileptic population also have serious *T. solium* infection in the form of NCC. (The number is much higher for Western blot immunoassay diagnosis, at 17.86%). This range is in accordance with other studies measuring the association between NCC and epilepsy, though this prevalence is smaller than others previously mentioned in this paper. This may be due to the small patient population as well as the focus on only epileptic patients rather than all patients with seizures. Further studies examining both an epileptic population and a non-epileptic, control population could help to clarify the link between NCC and epilepsy in Asia as well as provide more material for statistical analysis regarding the significance of the role of NCC in epilepsy. Also, additional studies looking at the location of both active and inactive cysts in NCC-diagnosed epileptic patients could further help elucidate the link between the worm infection and the neurological condition, and perhaps provide treatment guidance.

Currently, affordable and accurate diagnosis is not widely available to the majority of the world's population suffering from NCC. This study shows that the Western blot immunoassay method is a useful diagnostics tool for NCC. Extensions of this study to better perfect the use of the method with Asian patients could increase the method's efficacy. This study also addresses and supports previous studies that have associated epilepsy with NCC. Further studies pinpointing the location of cysts in a larger population of epileptic patients diagnosed with NCC could provide additional information about the link between the worm infection and the neurological condition.

3. Project II: Vaccine Development

3.1. Aim

The aim of this vaccine development study was two-fold. The first aim was to prepare a new edible vaccine using the novel protein, Tsol15 (493 plasmid). This was followed by completion of the second aim, to test two generations of transformed *A. thaliana* plants for the presence of a vaccine vector containing the highly immunogenic and well-characterized *T. solium* protein, Tsol18.

3.2. Materials and Methods

3.2.1. Preparation of vector

Preparation of competent E. coli *DH5α.* Single colonies of *E. coli* DH5α cells were grown in 5 separate tubes at 37°C in 10 ml LB media (Sigma Aldrich, USA) until the optical density reached 0.50AU at 600 nm. The tubes were centrifuged for 10 minutes, the supernatant removed, and 50 ml ice-cold 0.1M calcium chloride added to each tube. The cell solutions were kept on ice for 30 minutes before undergoing centrifugation at 5°C for 10 minutes. The supernatant was again removed, 200 ul (per every 50 ml cell solution) ice-cold 0.1M calcium chloride and 500 µl 50% glycerol were added to each tube and the tubes were stored on ice for 20 minutes to increase competency and then removed to -80°C for storage.

Transformation, preparation and digestion of pGEX-6p-1 plasmid. Ten microliters pGEX-6p-1 plasmid was added to 200 µl competent *E. coli* DH5α cells and kept on ice for 30 minutes. The solution was then heat-shocked at 42°C for 90 seconds, transferred to ice for an additional 2 minutes, and 800 µl LB media added. After 60 minutes incubation at 37°C, the tube was centrifuged at 13 000 rpm for 1 minute, half the supernatant removed, and the contents newly mixed. Two hundred microliters of the transformed bacteria solution was spread on 0.001% ampicillin plates and grown at 37°C for 12 hours. Successful transformation of *E. coli* DH5α with pGEX-6p-1 plasmid was indicated by growth on ampicillin plates.

Plasmid pGEX-6p-1 was purified from *E.coli* DH5α cells using the GenElute™ Plasmid Miniprep Kit by following the manufacturer's standard protocol (Sigma-Aldrich, Incorporated, USA). Successful transformation and purification of the cells was reaffirmed with digestion by Not1 according to standard protocol. Seventeen microliters of the purified product was added to 2 µl 10X FastDigest® Buffer and 1 µl Not1 (Fermentas Life Sciences, Canada) and incubated in a water bath at 37°C for 90 minutes. Success of the digestion was verified by running 5 µl of the digested product with 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel. Digestion with enzymes BamH1 and EcoRI and their associated buffer was also performed following standard protocol provided by the manufacturer (Fermentas Life Sciences, Canada). Sixteen microliters of the non-digested, purified PCR product was added to 2 µl 10X FastDigest® Buffer, 1 µl BamH1, and 1 µl EcoR1 (Fermentas Life Sciences, Canada) and incubated in a water bath at 37°C for 90 minutes. Success of the digestion was verified by running 5 µl of the digested product with 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel.

3.2.2. Preparation of insert

Amplification, purification, and digestion of 493 plasmid. Polymerase chain reaction (PCR) was performed on two samples of the 493 plasmid, one comprised of *E. coli* with the plasmid and the other the pure plasmid in solution. Each PCR cocktail consisted of 16.4 µl Milli-Q water, 2.5 µl 10X AmpliTaq Gold® Buffer (Applied Biosystems Incorporated, USA), 1.5 µl 25mM $MgCl_2$ (Applied Biosystems Incorporated, USA), 0.5 µl 100U AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems Incorporated, USA), 0.5 µl 10mM dNTP (Invitrogen Limited, United Kingdom), 1.3 µl 493rev primer (5'-CGGGAATTCCGCAAAGAATACCGCCAT GCAA-3'; Eurofins, France), 1.3 µl 493for primer (5'-CTGGGATCCATGAATGCAAGTCAAATG CTAG-3'; Eurofins, France), and 1 µl of either *E. coli* with 493 plasmid, pure plasmid, or Milli-Q water (to serve as the negative control). The protocol followed was 5 minutes at 96°C, 35 cycles of 45 seconds at 96°C, 45 seconds at 52°C, and 80 seconds at 72°C, and finally 7 minutes at 72°C before storage at 4°C. Amplification was verified by running 5 µl of the PCR products with 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel. Purification of the PCR products was performed by following the standard protocol for using a microcentrifuge with the QIAquick® PCR Purification Kit (Qiagen, Germany). Forty microliters of product was mixed with 200 µl provided PB buffer, put into a provided spin column, and centrifuged for 60 seconds. The flow-through was discarded and 750 µl provided PE buffer was added before centrifugation for 60 seconds. Next, the flow-through was discarded again and the spin column centrifuged an additional 60 seconds. The column was moved to a clean microcentrifuge tube and 40 µl provided EB elution buffer was added to the center of the spin column. After 90 seconds, the column was centrifuged for 60 seconds and then the flow-through kept as purified product.

Digestion of the purified PCR products using enzymes EcoR1 and BamH1 was performed following standard protocol provided by the manufacturer (Fermentas Life Sciences, Canada). Thirty-four microliters of the purified PCR product was added to 4 µl FastDigest® 10X Buffer,1 µl EcoRI, and 1 µl BamH1 (Fermentas Life Sciences, Canada) and kept in a water bath at 37°C for 90 minutes. Success of the digestion was verified by running 5 µl of the digested product with 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel. Results were compared against 1 µl of GeneRuler™ Express DNA Ladder (Fermentas Life Sciences, Canada).

3.2.3. Preparation of vector/insert construct

Purification of digested pGEX-6p-1 plasmid and digested 493 plasmid . Purification of both the digested pGEX-6p-1 vector and the digested 493 plasmid was performed using the QIAquick® PCR Purification Kit and following the standard protocol for purification with a microcentrifuge. Thirty microliters of each sample were mixed individually with 150 µl provided PB buffer, put into provided spin columns, and centrifuged for 60 seconds. The flow-through was discarded and 750 µl provided PE buffer was added before centrifugation for 60 seconds. Next, the flow-through was discarded again and the spin column centrifuged an additional 60 seconds. The columns were moved to clean microcentrifuge tubes and 40 µl provided EB elution buffer was added to the center of the spin column. After 90 seconds, the columns were centrifuged for 60 seconds and then the flow-through kept as purified, digested pGEX-6p-1 vector and purified, digested 493 plasmid.

Determination of DNA concentration of purified pGEX-6p-1 plasmid and purified 493 plasmid. Standard protocol for the SmartSpec™ Plus Spectrophotometer (Bio-Rad Laboratories Limited, United Kingdom) and NanoDrop® ND-1000 (NanoDrop Technologies,

USA) was followed to determine the DNA concentration of the two digested and purified samples. One milliliter Milli-Q water was used as the blank.

Ligation of digested pGEX-6p-1 vector with digested 493 plasmid. According to the concentrations determined in step 2.5, Milli-Q water, the purified pGEX-6p-1 and 493 plasmids from step 2.4, T4 DNA ligase (Invitrogen Limited, United Kingdom), 5X T4 DNA ligase buffer (Invitrogen Limited, United Kingdom), and 10 mM dATP (Invitrogen Limited, United Kingdom) were added to 3 tubes labeled A, B, and C to give insert-to-vector (493 plasmid: pGEX-6p-1 plasmid; i:v) ratios of 1:1, 3:1, and 0:1, as shown below:

	i:v	**MQ water**	**493 (i)**	**pGEX-6p-1 (v)**	**Ligase**	**Ligase buffer**	**dATP**	**Total**
A	1:1	3 µl	2 µl	1 µl	1 µl	2 µl	1 µl	10 µl
B	3:1	0 µl	5 µl	1 µl	1 µl	2 µl	1 µl	10 µl
C	0:1	5 µl	0 µl	1 µl	1 µl	2 µl	1 µl	10 µl

The tubes were gently mixed and incubated at room temperature for 7 minutes under standard rapid ligation protocol for T4 DNA ligase (Invitrogen Limited, United Kingdom).

Transformation of TOPO® One Shot® chemically competent cells with ligated plasmid. Immediately following completion of the ligation, 5 µl from each ligation tube A, B, and C was added to separate tubes of TOPO® OneShot® chemically competent cells (Invitrogen Limited, United Kingdom) and gently mixed. The tubes were incubated on ice for 30 minutes and then heat-shocked at 42˚C for 30 seconds. Then, the tubes were immediately transferred back to ice and 250 µl S.O.C. medium (Invitrogen Limited, United Kingdom) was added to each tube. The three tubes were shaken horizontally at 37˚C and 200 rpm for 60 minutes. After incubation, 20 µl S.O.C. medium was added to each tube and 100 µl from each was spread onto pre-warmed 0.001% ampicillin plates. The plates were incubated at 37°C overnight. Successful transformation of TOPO® OneShot® chemically competent cells with the ligated plasmid was indicated by growth on ampicillin plates followed by single-colony digestion using Not1 and Sal1 as well as BamH1 and EcoR1. Digestion was performed after purification with the GenElute™ Plasmid Miniprep Kit by following the manufacturer's standard protocol (Sigma-Aldrich, Incorporated, USA). Digestion with enzymes BamH1/EcoR1, Not1/Sal1, and their associated buffer was performed following standard protocol provided by the manufacturer (Fermentas Life Sciences, Canada). Sixteen microliters of the purified PCR product was added to 2 µl 10X FastDigest® Buffer, 1 µl BamH1 (or Not1), and 1 µl EcoR1 (or Sal1) (Fermentas Life Sciences, Canada) and incubated in a water bath at 37°C for 2 hours. Success of the digestion was verified by running 5 µl of the digested product with 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel. Results were compared against 1 µl of GeneRuler™ Express DNA Ladder (Fermentas Life Sciences, Canada).

3.2.4. Verification of construct success

Amplification and purification of 493pGEX-6p-1 construct. Five milliliters of 493pGEX-6p-1 construct in transformed TOPO® OneShot® chemically competent cells was grown overnight to saturation in 45 ml LB media (Sigma Aldrich, USA). The solution was centrifuged at 300 rpm for 15 minutes and the supernatant removed. Purification was performed using the QIAGEN® Plasmid Maxi Prep Kit and following the standard protocol for the Plasmid Midi prep purification (Qiagen, Germany). The pellet was resuspended in 4 ml Buffer P1 (resuspension solution). Four milliliters of Buffer P2 (lysis solution) was added and the

solution mixed thoroughly by vigorously inverting 6 times. After incubation at room temperature for 5 minutes, 4 ml of ice-cold Buffer P3 (neutralization solution) was added and the solution immediately inverted before 15 minutes incubation on ice. The solution was mixed again by inverting 4 times and then centrifuged at 4°C for 30 minutes. After centrifugation, the supernatant was immediately removed to a sterile tube and centrifuged at 4°C for 15 minutes. Again, after centrifugation, the supernatant was removed to another sterile tube and 240 µl saved for later analysis. The supernatant was added to a QIAGEN-tip 500 which had been previously equilibrated with 10 ml provided Buffer QBT (equilibration solution) and allowed to flow into a sterile tube by gravity. Two hundred and forty microliters of the flow-through was saved for later analysis. The remaining flow-through was discarded and the QIAGEN-tip 500 washed twice with 10 ml provided Buffer QC (wash solution). Both times, the buffer was allowed to flow by gravity. A 400 µl aliquot of the flow-through was kept for later analysis and the tip placed over a new sterile tube. Five milliliters of provided Buffer QE (elution solution) was added to the tip and elution by gravity occurred. One hundred microliters of the eluate was set aside for later analysis and the DNA in the remaining eluate was precipitated using standard ethanol precipitation protocol as described in the product manual.

Room temperature isopropanol was added to the eluted DNA in 0.7x volume (here, 3.5 ml isopropanol) and mixed by inverting 4 times. The solution was centrifuged at 4 500 rpm for 30 minutes at 4°C and the supernatant carefully removed. The remaining pellet was washed with 2 ml room temperature 99.5% ethanol and centrifuged at 4 500 rpm and 4°C for 10 minutes. Again, the supernatant was carefully removed and the pellet air-dried for 15 minutes before resuspension in 50 µl Milli-Q water.

Tranformation, purification, and digestion of competent E. coli *BL21 cells with ligated plasmid.* Ten microliters of ligated plasmids in tubes A, B, and C were separately added to 130 µl competent *E. coli* BL21 cells and incubated on a shaker at 37ºC for 90 minutes. One hundred microliters from each tube were grown on corresponding 0.001% ampicillin plates overnight at 37ºC. Five single colonies from plate C were grown, shaking, at 37ºC in 2 ml LB media with 2 µl ampicillin until OD reached 0.5; concurrently, a PCR of each of the 5 colonies was also performed as described in *2.2*. (The protocol is identical except that instead of rather than using 1 µl sample, pipette tips containing the colony of interest were stirred into the PCR cocktail before being dropped into the LB/ampicillin media for growth.) A 1% agarose gel was run with 5 µl PCR product and 2 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom). The positive samples plus one negative control were purified as performed previously using the GenElute™ Plasmid Miniprep Kit and following the manufacturer's standard protocol (Sigma-Aldrich, Incorporated, USA).

Digestion of the purified products was performed using BamH1 and Kpn1. Sixteen microliters of each product, 2 µl 10X FastDigest® Buffer, 1 µl BamH1, and 1 µl Kpn1 (Fermentas Life Sciences, Canada) were incubated at 37°C for 2 hours. Successful digestion was verified by running 2.5 µl sample with 1 µl 10X BlueJuice™ Gel Loading Buffer (Invitrogen Limited, United Kingdom) on a 1% agarose electrophoresis gel. Results were compared against 1 µl of GeneRuler™ Express DNA Ladder (Fermentas Life Sciences, Canada).

Induced expression of GST fusion protein. To determine the optimal length of GST expression by IPTG, a time course was established. Five milliliters of *E. coli* BL21 containing the 493pGEX-6p-1 construct was added to 45 ml LB and incubated at 37°C on a shaker until the optical density reached 0.5AU. A 10 µl aliquot was removed and 0.1 mM IPTG added. At 1 hour, 2 hours, 3 hours, and 4 hours, 10 µl of the sample was removed. An equal amount of 2x

SDS loading buffer was added to each aliquot[6] before incubation at 95°C for 4 minutes. Twenty microliters of each was run on a 12% SDS-Page gel[7] at 200 V. Ten microliters of protein ladder (Protein Molecular Weight Standard, broad range MW 6500-205 000, Amersham Biosciences UK Limited, United Kingdom) was also used.

Five milliliters of *E. coli* BL21 containing the 493pGEX-6p-1 construct was added to 45 ml LB and incubated at 37°C on a shaker until the optical density reached 0.5AU. Expression with 0.1 mM IPTG (Sigma Aldrich, USA) at 37°C and shaking for 2 hours was performed, and then the culture was centrifuged at 300 rpm for 20 minutes at 23°C. The supernatant was removed and the pellet stored at -20°C. Two 50 µl aliquots of the culture were taken just before addition of the IPTG and just before centrifugation. Each aliquot was added to 50 µl 2x SDS loading buffer[8] and briefly vortexed and centrifuged before incubation at 95°C for 4 minutes. Both aliquots were run on a 12% SDS-Page gel[9] at 20 µl per well and 200 V. Ten microliters of protein ladder (Protein Molecular Weight Standard, broad range MW 6500-205 000, Amersham Biosciences UK Limited, United Kingdom) was also used.

3.2.5. Transformation of *Arabidopsis thaliana* with Tsol18 and confirmation of its success with DNA and protein analyses

At Örebro University, *Agrobacterium tumefaciens*-mediated gene transfer of a specially-constructed Tsol18 vector (with BASTA resistant marker) was performed on the T0 generation of *Arabidopsis thaliana*. Seeds were harvested from the T0 plants and grown on plates containing BASTA; the seeds that grew became the T1 generation. After 2 weeks, plants were removed from the BASTA plates to soil to continue to grow. Seeds were harvested from the T1 plants and grown again on BASTA plates to give the T2 generation. The entire transformation process was performed twice.

DNA extraction on each of the 4 T1 generation plants from the first transformation and each of the 22 T1 generation plants from the second transformation was performed using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, USA). A portion of one leaf was removed from each plant using forceps and scissors and placed into separate tubes containing 100 µl Extraction Solution so that the leaf sample was entirely submerged in the solution. The forceps and scissors were sterilized with 96% ethanol between contact with different plants. Each tube containing a leaf sample was vortexed and incubated at 95°C for 10 minutes before the addition of 100 µl Dilution Solution. The samples were vortexed once more and stored at 4°C. To verify success of the method, two leaf samples were taken at random and tested for DNA concentration and purification, as performed previously using the NanoDrop® ND1000.

PCR amplification was performed as recommended in the REDExtract-N-Amp™ Plant PCR Kit: 10 µl REDExtract-N-Amp™ PCR ReadyMix™, 1 µl TsolF primer, 1 µl 35sr primer (5'-CTTATCGGGAAACTACTCACACATT-3'), and 8 µl of leaf extract were run on a GeneAmp® PCR System 9700 (Applied Biosystems, United Kingdom) with 5 minutes at 94°C, 35 cycles of 45 seconds at 94°C, 45 seconds at 52°C, and 80 seconds at 72°C, followed by 7 minutes at 72°C and a hold at 4°C. One microliter of a positive sample containing the Tsol18 plasmid (Irina Kalbina, Örebro University, Sweden) plus 7 µl Milli-Q water was used as the positive control in place of 8 µl plant extract, while 8 µl Milli-Q water similarly served as the negative control. To verify successful transformation of the plants, 5 µl of each PCR product was run

[6] See Appendix 7.6.
[7] See Appendix 7.1. and 7.2.
[8] See Appendix 7.6.
[9] See Appendix 7.1 and 7.2.

on a 1% agarose electrophoresis gel at 100 V. Results were compared against 1 µl of GeneRuler™ Express DNA Ladder (Fermentas Life Sciences, Canada).

Verification of Tsol18 presence in T2 generation Arabidopsis thaliana. With Irina Kalbina at Örebro University, seeds from transformed (BASTA-resistant) *Arabidopsis thaliana* were grown on a Petri dish with BASTA medium. A plate containing the negative control, a transgenic (BASTA-resistant) *A. thaliana* containing the MOMP protein from Chlamydia, was grown simultaneously under identical conditions.

Leaves were harvested from the plants on day 15 and stored at -20°C. Five leaves were added to 99 µl 0.1 M Tris-HCl (pH 7.6) plus 1 µl protease inhibitor (Protease Inhibitor Cocktail for use with mammalian cell and tissue extracts, DMSO solution; Sigma® Life Science, USA) and manually homogenized on ice. The solutions were centrifuged at 13 000 rpm for 5 minutes and the supernatant transferred to a new tube. One hundred microliters of 1x SDS loading buffer[10] was added to each supernatant and both solutions were vortexed and then centrifuged briefly before incubation for 4 minutes at 95°C. A positive control for the process, using a homogenized *T. solium* cyst from Nicaragua, was also prepared by adding equal amounts 2x SDS loading buffer[11] to homogenized cyst and incubating at 95°C for 4 minutes. Each plant sample was run on a 12% SDS-Page gel[12] at 20 µl per well and the cyst sample at 6 µl per well. Ten microliters of protein ladder (Protein Molecular Weight Standard, broad range MW 6500-205000, Amersham Biosciences UK Limited, United Kingdom) was also used.

A portion of the gel was stained with Coomassie blue stain (BioSafe™ Coomassie G-250 Stain, BioRad Laboratories Incorporated, USA) to verify success of the gel; the remainder was used for Western Blot following standard semi-dry electrophoretic transfer protocol. Three sheets of absorbent filter paper were cut to match the size of the gels and dipped in semi-dry transfer buffer[13] and placed on the semi-dry apparatus (NovaBlot with Electrophoresis Power Supply EPS-3500XL; Amersham Pharmacia Biotech, United Kingdom). One sheet of Hybond™ -ECL membrane (Amersham Pharmacia Biotech, United Kingdom) also cut to match the size of the gel was carefully submerged in distilled water, equilibrated in semi-dry transfer buffer[14] for 10 minutes, and placed on top of the filter paper. The gel was placed on top of the nitrocellulose membrane and three additional pieces of filter paper (also dipped in semi-dry transfer buffer) were placed on top. The apparatus was set to run for one hour with 90 mA as the limiting factor.

Blocking was performed in 5% nonfat milk buffer (5 g nonfat milk in 100 ml TBS) for one hour at room temperature on an agitator (IKA® KS 130 Basic, IKA®-Werke GMBH & Co.KG, Germany). Separate lanes were cut from the membrane and placed in 3 separate Petri dishes: the positive control, negative control, and Tsol18 sample in one containing 10 ml of primary antibody solution (concentration 1:500; Anti-Tsol18 (from rabbit); from Irina Kalbina, Örebro University), the positive control in one containing 10 ml of human primary antibody solution (concentration 1:5000, or 2 µl positive sera from a Nicaraguan patient), and another Tsol18 sample in one containing milk buffer only. All samples were agitated at room temperature for 60 minutes or overnight at 4°C.

[10] See Appendix 7.6.
[11] See Appendix 7.6.
[12] See Appendix 7.1 and 7.2.
[13] See Appendix 7.4.
[14] See Appendix 7.4.

All samples were washed three times at room temperature with TBS + 0.05% PlusOne Triton X-100 (GE Healthcare, Uppsala, Sweden) solution for twenty minutes per wash. All samples were agitated during washing.

Ten milliliters of secondary antibodies (concentration 1:1000) in 10 ml 5% nonfat milk were added to 3 separate Petri dishes following washing: the positive control, negative control, and Tsol18 sample in one containing anti-rabbit IgG secondary antibodies (Anti-rabbit IgG Horseradish Peroxidase-linked whole antibody (from donkey); GE Healthcare Limited, United Kingdom), the positive control in one also containing anti-rabbit IgG secondary antibodies, and the Tsol18 sample in one containing anti-human secondary antibodies (Anti-human Ig Horseradish Peroxidase-linked whole antibody (from sheep); Amersham Biosciences, United Kingdom). The samples were agitated in separate Petri dishes at room temperature. After 60 minutes, the samples were washed three times for twenty minutes as performed previously.

Chemiluminescence detection was performed through development of film exposed to the washed nitrocellulose membrane. Standard protocol for the Amersham™ ECL Plus Western Blotting System (GE Healthcare, United Kingdom) was followed: One milliliter Solution A (Lumigen™ PS-3 detection reagent) and 25 µl Solution B (Lumigen™ PS-3 detection reagent) were mixed in a 1.5 ml tube. The nitrocellulose membrane was placed on the cassette and washed with the Solution A/Solution B mix so that the entire membrane was covered; excess solution mix was removed with a dry cloth. A clear film was placed over the soaked membrane and once in the dark room, the Amersham Hyperfilm™ ECL developing film (GE Healthcare Limited, United Kingdom) was exposed to the membranes for a variable amount of time and standard developing protocol using the Curix 60 (Agfa Healthcare, Belgium) was followed.

Petri Dish	Contents	Primary Antibodies	Secondary Antibodies
1	Positive control[*] Negative control[¥] Tsol18 sample	Anti-Tsol18 (rabbit), [1:500]	Anti-rabbit IgG Horseradish Peroxidase-linked whole antibody (from donkey), [1:1000]
2	Positive control	None	(As above)
3	Tsol18 sample	Positive sera (human) [1:5000]	Anti-human Ig Horseradish Peroxidase-linked whole antibody (from sheep)
[*] Positive control: Nicaraguan cyst [¥] Negative control: MOMP plant			

Table 2. Summary of primary and secondary antibody addition.

3.3. Results

A novel vaccine construct was created by inserting the novel protein 493 plasmid into a pGEX-6p-1 vector. Figure 7 shows the DNA sequence of the final construct, including the relevant area of the vector (in blue) as well as the 493 plasmid (in red). The dotted red line indicates a proposed location of a polyhistidine-tag insert to aid in purification. This figure shows that the plasmid-vector construct yields a complete running sequence.

3.3.1. Creation of recombinant 493pGEX-6p-1 plasmid.

Figure 7. Relevant portions of the 493pGEX-6p-1 construct with important restriction sites shown. The blue line marks the open reading frame of the pGEX-6p-1 vector, while the solid red line shows the 493 plasmid. The dotted red line is a proposed polyhistidine-tag insert.

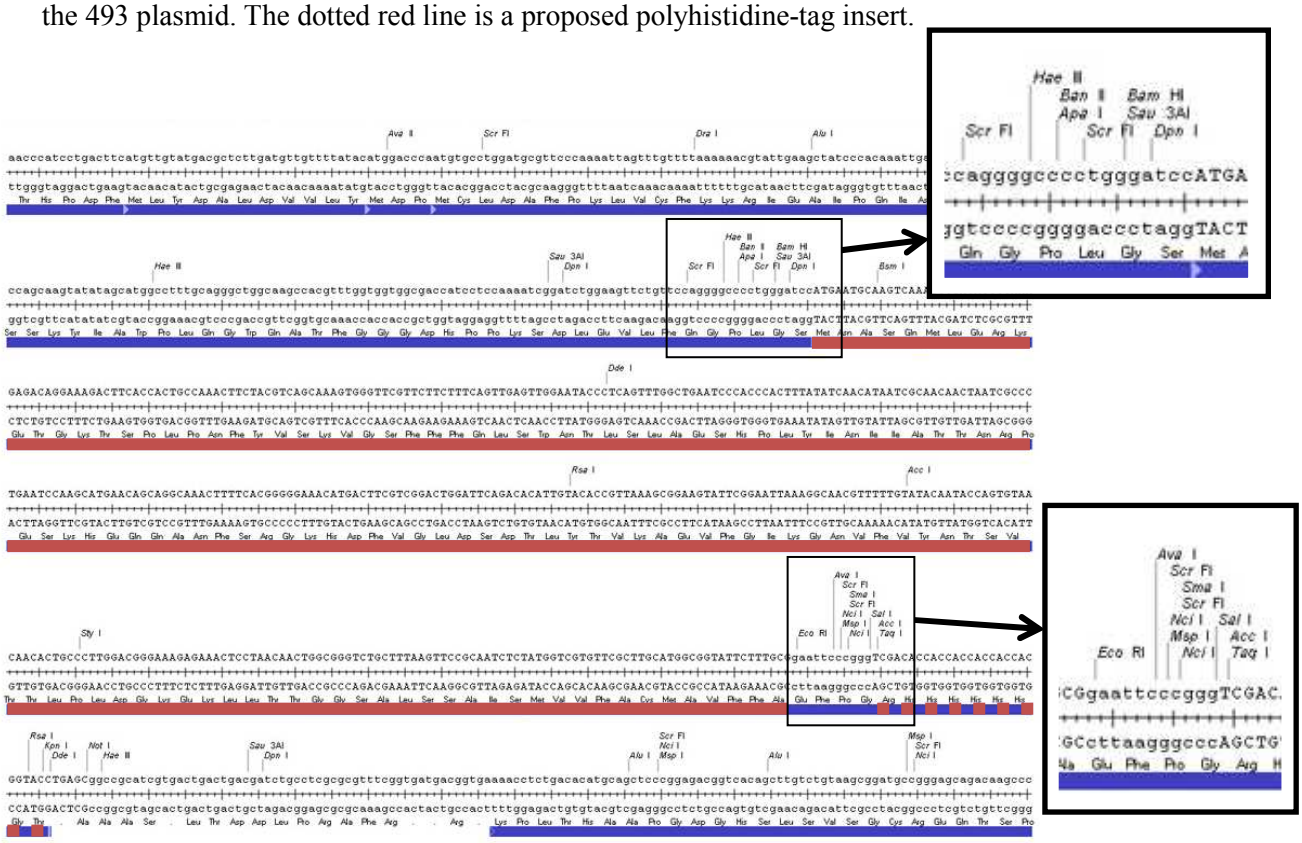


Figure 8 shows verification of the digestion of the 493 plasmid and the pGEX-6p-1 vector in preparation for their ligation. This figure shows that both the 493 plasmid insert and the pGEX-6p-1 vector are pure by gel analysis and of the expected sizes.

Figure 8. Verification of the digestion of PCR amplified and purified 493 plasmid (*i*) and purified pGEX-6p-1 vector (*v*) in transformed *E. coli* DH5α cells, grown in LB media and 0.001% ampicillin. The insert showed band length of about 475 base pairs and the vector showed length of about 5000 base pairs, as expected.

QuickTime™ and a decompressor are needed to see this picture.

Figure 9 shows the digestion of single colonies of cells transformed with the 493pGEX-6p-1 construct. Although the construct contains an ampicillin-resistance region, Figure 9a shows that some of the colonies that grew on the 0.001% ampicillin plate were not positive for the entire contruct, but rather for only the pGEX-6p-1 vector. Figure 9b reconfirms the results of Figure 9a by verifying that colonies 2, 3, 4, 6, 7, and 9 are positive for the 493 plasmid insert by PCR amplification using 493 plasmid-specific primers. This indicates that the construction of the vector was successful.

Figure 9. Digestion of the single colonies of transformed TOPO® One Shot ® chemically competent cells with 493pGEX-6p-1 construct, using Bam H1 and Eco R1. Single colonies were chosen from the ampicillin plate with ligation ratio 3:1 (insert:vector) and amplified using PCR (*a*). Colonies 2, 3, 4, 6, and 7 were reverified with digestion to be positive for successful ligation (*b*). Sample "C" is the negative PCR control and sample 9 is the negative control for growth on ampicillin plates.

Figure 9a

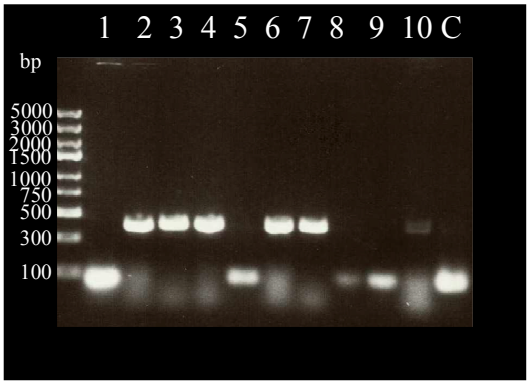


Figure 9b

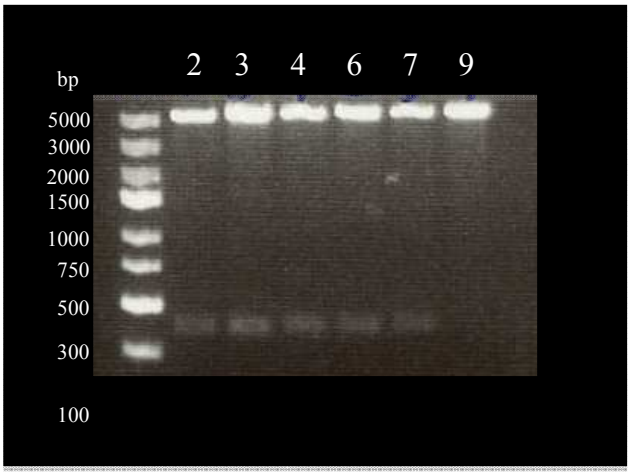
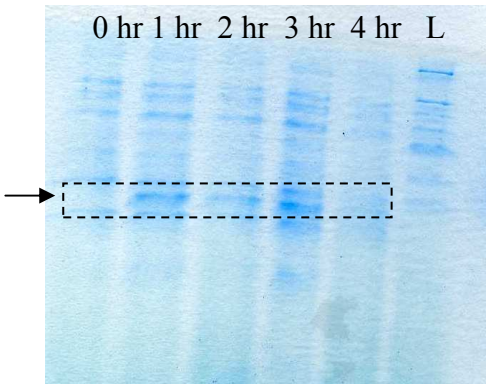


Figure 10a



42 kDa

10b

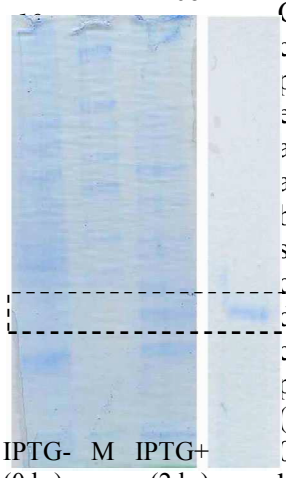


Figure 10. Induced expression of GST fusion protein. A time course over 4 hours was performed, with similar GST expression induced at hours 1, 2, and 3 and worse GST expression at 4 hours. The ladder is denoted by "L" and all samples are shown after Coomassie staining of a 10% gel (*a*). A definitive difference between sample containing the GST fusion protein before (at right) and after (at left) 2 hours induction with 0.1mM IPTG is shown in (*b*). A band of 42 kDa size is observed after induction, indicating that the GST fusion protein is expressed. Figure (c) shows the purified construct on beads; also at size 42 kDa.

Figure 10 shows that the GST fusion protein, or the part of the 493pGEX-6p-1 construct that is expressed with the lac operon region is no longer repressed, can be visualized using Western blot. Figure 10a shows a time course that allowed the optimal amount of time for incubation of the construct with 0.1 mM IPTG to be determined; two hours incubation at 37°C was determined to be the optimal conditions. Figure 10b shows that the GST fusion protein is only expressed when incubated with 0.1 mM IPTG and that the size of the protein approximately 42 kDa, as expected. It is also shown in Figure 10b that one potential method of purification of the 493 plasmid (Tsol15 protein) from the GST fusion protein is using Glutathione Sepharose 4B beads, which bind to the GST protein and allow site-specific cleavage with a specific protease so that the Tsol15 protein can be removed to the supernatant. In this figure, the protease was not used to cleave the Tsol15 protein from the GST protein so it is shown that the GST fusion protein does bind to the beads, at the size of 42 kDa (the expected size of the entire construct).

3.3.2. Verification of the transformation of T1 generation A. thaliana *with Tsol18 recombinant plasmid.*

Figure 11 shows PCR amplification of the 22 *A. thaliana* plants that were grown from seeds of an *A. thaliana* plant that was transformed with a Tsol18 vaccine construct. It is shown that only 5 of the 22 plants were positive for the Tsol18 construct after PCR amplification using Tsol18-specific primers. Further analysis showed that sample 4 may also be positive for the construct but this is not pictured. This figure indicates that not all offspring of the original transformed plant are positive for transformation (T1 generation), and therefore allows for selective production of the T2 generation of Tsol18-transformed plants.

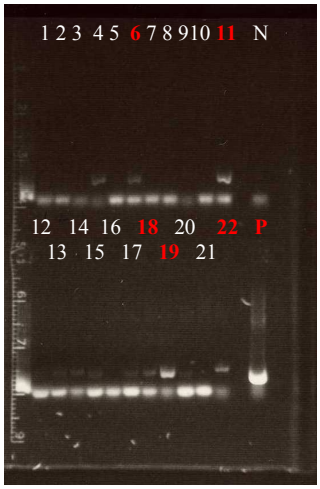


Figure 11. Leaves from 22 T1 *A. thaliana* plants were tested for transformation of the Tsol18 vector. "N" denotes the negative sample (Milli-Q water) and "P" denotes the positive sample (Tsol18 plasmid). Samples 6, 1, 18, 19, and 22 were considered positive; one additional sample (4) was also considered positive by further PCR analysis (not pictured).

3.3.3. Verification of retained Tsol18 recombinant plasmid in T2 generation A. thaliana.

Figures 12 and 13 show that detection of the Tsol18 protein is possible using the Western blot immunoassay method, and seems to be detected in the T2 generation. In Figure 12, the anti-Tsol18 antibody used in this experiment was shown to be viable and specific to Tsol18. Furthermore, Tsol18 is shown to remain undetected with anti-GST antibodies are used as part of the Western blot immunoassay procedure. In Figure 13, the single surviving plant from the T2 generation of Tsol18-transformed *A. thaliana* is shown to perhaps be positive for the Tsol18 protein in its leaves and stems. The band is difficult to visualize and therefore these results are considered inconclusive but hopeful. The MOMP plant, which contains a protein specific to Chlamydia, is negative by this same analysis, as expected.

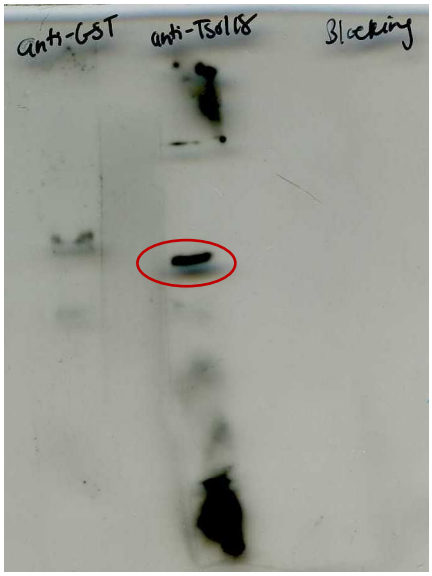


Figure 12

Figure 12. Western blot immunoassay showing that the anti-Tsol18 antibody was viable. The band circled in red shows that anti-Tsol18 primary antibody will detect Tsol18 plasmid.

Figure 13. Results from Western blot immunoassay analysis of (1) the MOMP plant, (2) the negative control, and (3) the Tsol18 plant. The red arrow shows a potential positive band around of approximately 18 kDa in size from the Tsol18 plant, as would be expected, but missing from the MOMP plant and the negative control.

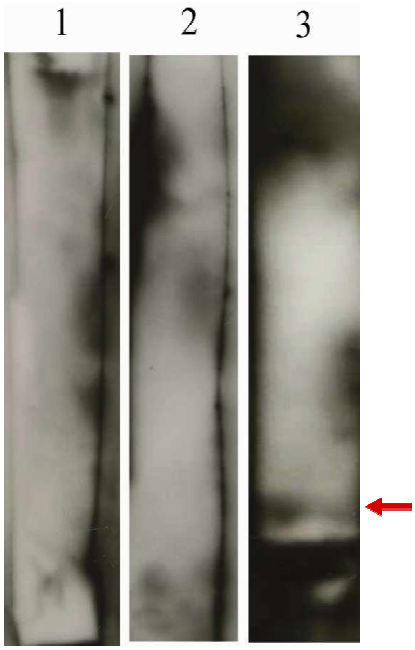


Figure 13

3.4. Discussion

A novel *T. solium* oncosphere protein was inserted into a pGEX plasmid and expressed. This was shown using PCR, restriction site digestion, and SDS-Page analyses. Furthermore, two generations of *A. thaliana* plants previously transformed with the immunoreactive *T. solium* oncosphere protein Tsol18 were shown by PCR amplification as well as potentially by the Western blot immunoassay method to be positive for a vector containing this protein. Therefore, this study shows that an edible vaccine candidate consisting of a plant with an inserted *T. solium* protein can be created.

Additional analysis to conclusively determine if the studied T2 generation plant contained the Tsol18 plasmid must be performed. It is possible that the plant expresses the protein in a very small amount, or that the expression differs among different parts of the plant. Testing the stems for the presence of the plasmid (as performed by Lindh *et al.* 2008) could yield better results.

In general, further steps must be taken in order to support the use of this construct as a porcine vaccine. First, the transgenic plant must be shown to elicit an immune response in mice. More transgenic *A. thaliana* must be grown and an experiment that tests its efficacy in a controlled environment must be designed and implemented. The immune response, as measured by antibody levels, must be determined in mice and potential problems such as oral tolerance or adverse effects must be monitored. Furthermore, many more generations of the transgenic *A. thaliana* must be grown in order to determine if gene silencing or similar situations occur with time.

An important step that was not completed during this project work is purification of the novel protein in the vector-insert construct. Gluthathione S-transferase (GST) is a protein from *Schistosoma japonicum*, a flatworm, and when its expression in *E. coli* is induced with IPTG, the product is a fusion protein with GST at one end and the inserted protein (here, Tsol15 or the 493 plasmid) at the other. Purification of Tsol15 from the GST fusion protein would allow for ease of reproduction of the protein of interest and also must be performed in order to test

its immunogenicity with patient sera; this is because many patients (particularly in Africa) with NCC infection may also have schistosomiasis, and therefore antibodies to the *Schistosoma* GST protein would be present in their sera and act as a confounding factor in this study. The recommended procedure for purifying the protein involves the use of Glutathione Sepharose 4B beads, which bind to the GST part of the fusion protein. A site-specific protease is then added to the beads in a buffer solution, and the protein of interest is cleaved so that the GST remains attached to the beads and the protein of interest can be found in the supernatant. This was attempted several times during the course of the project, and limited success was shown. In most cases, the method itself was shown to work by SDS-Page analysis but the purified protein of interest was present in small amounts and never truly "pure" but rather, only cleaved. Several factors may contribute to this. Simple difficulty separating the supernatant from the beads resulted in a substantial amount of supernatant remaining unremoved, thereby rendering the bead solution impure. Furthermore, it is possible that the protein of interest unspecifically bound to the GST beads after cleavage; washing the beads with a saturated salt solution could help release the purified Tsol15 protein while the GST protein remains attached. Optimizing the site-specific cleavage by altering the ratio of protease to protein may also provide greater success of the purification.

The insertion of a polyhistidine-tag for use as another method of purification could also be performed. If added after the protein of interest as shown in Figure 1 by using different restriction enzymes (that is, Not 1 and Sal 1 rather than Bam H1 and Eco R1), the protein of interest could easily be removed and exchanged for other proteins of interest in the future while the polyhistidine-tag remains intact. Purification by polyhistidine-tag occurs due to histidine affinity for nickel, which allows specifically-cleaved solutions to be sorted through a nickel column so that the polyhistidine-tag with the protein of interest remains attached to the nickel column without the GST protein.

This study represents the beginning of the creation of a potentially effective edible vaccine. "Proof-of-principle" is illustrated by detection of the protein in leaves of BASTA resistant plants, but further experiments testing the immune response of mice that have eaten the transgenic plants must be conducted. Furthermore, continued work resulting in the transformation of *A. thaliana* with the newly-constructed vector containing Tsol15 must also occur. If both plants show success at eliciting immune responses in mice, the next step would be the transformation of larger plants (such as carrots) so that a realistic amount of plant material could be grown and fed to pigs in an experimental porcine trial for this edible vaccine.

4. Conclusion

This project work represents the beginning of a promising path towards the development of two edible vaccines against the porcine tapeworm, *Taenia solium*, shows that Western blot immunoassay is a reliable method of diagnostics in Vietnam, and supports findings from previous studies that associate the occurrence of epilepsy with *T. solium* infection in humans. Additional steps could be taken in each of these areas to further advance development of the vaccine and increase knowledge of diagnostics success of patients infected with the tapeworm.

Twenty-eight epileptic patients from Vietnam were used as subjects of the first part of this study; only two showed signs of *T. solium* infection using brain imaging methods. However, both of those patients showed a specific band of about 80-100 kDa in length. Additional research characterizing this result and determining whether it denotes a novel or important protein could expose an Asia-specific cysticerci antibody with potential diagnostics and vaccine development applications. Testing the Vietnamese patient sera with homogenized Asian cysticerci could also provide more clues to the antibody's (and antigen's) potential, as could testing homogenized Asian cysticerci with African and Latin American patient sera. Furthermore, using the Western blot immunoassay method to test the Vietnamese patient sera with a single protein or a purified set of several proteins (as opposed to the numerous proteins present in crude homogenized cysticerci) could provide more information about the success of the method. Finally, using negative and positive sera from Vietnamese patients rather than Nicaraguan patients could provide information about the reaction's importance. It could also be interesting to use sera from patients without any history of epilepsy, to see if there is a difference between band patterns of epileptic and non-epileptic NCC patients. It could be that the results observed represent a NCC-and-epilepsy-specific reaction rather than an Asia-specific reaction.

Results from the vaccine development portion of this study indicate that *A. thaliana* can be transformed with a vector containing a specific *T. solium* protein and show evidence by DNA and potentially protein analyses in multiple generations. An additional vector containing a novel protein of interest was created and further steps to prepare the vector for *A. tumefaciens*-mediated gene transfer to *A. thaliana* could be completed in the near future. This includes purifying the vector, transforming *A. thaliana*, growing multiple generations of the plant, and analyzing each one for the presence of the protein. Feeding the transformed plant to mice, much as performed by Lindh *et al.* 2008, would be the next step for both edible vaccine candidates, followed by the transformation of the vector into a larger plant (such as carrots) for use in trials with pigs.

More work could also focus on the characterization of the protein of interest in this study. The Tsol15 protein (or 493 plasmid) is a novel protein, with homology to a gene family of *T. ovis*. Little else is known about the protein. Additional studies to determine the protein's function and structure should be performed to characterize this novel antigen and perhaps provide more information regarding the pathology of CC.

One of the general criticisms for edible vaccines is the development of oral tolerance rather than the stimulation of mucosal immunity. More information must be collected in order to evaluate the impact of oral tolerance on an edible porcine vaccine; as previously mentioned, no study addressing this currently exists. A review article by Holmgren and Czerkinsky addresses the mucosal immune response to vaccines and points out that the mucosal immune system is a "highly compartmentalized immunological system [that] functions essentially

independent from the systemic immune apparatus" (2005). Differential expression of mucosal immune responses depends on the area of vaccine introduction and the authors show that an oral route of vaccination leads to IgA immune responses not only in the salivary glands but also in the stomach, intestines/gut, and even the mammary glands. Tolerance, they write, can result to protect from harmless antigens and forms from activation-induced cell death, anergy of antigen-specific T-cells presented in large amounts, and the induction of regulatory T-cells. The use of an edible vaccine is a complex system that requires more understanding of the delicate balance between inducing a response (which may call for the use of an adjuvant) and preventing oral tolerance.

That the mucosal immune system is compartmentalized warrants further research in regards to both an edible porcine *T. solium* vaccine and human diagnostics. If the mucosal immune system is as independent of the systemic immune system as Holmgren and Czerkinsky write, perhaps saliva – a surface of that system – could provide better diagnostics results than sera. Furthermore, since the oral route of administration also stimulates an immune response in the mammary glands in humans, research to determine whether an oral vaccine stimulates an immune response which can be passed from sow to piglet also should be performed.

The aims of this project were to investigate diagnostic methods and the link between <ncc and epilepsy in Vietnamese patients, develop the first steps of an edible vaccine using a novel *T. solium* cysticerci protein, and determine the viability of a previously-created edible vaccine containing a well-characterized *T. solium* cysticerci protein. The aims were completed in full. However, additional work to conclusively determine the presence of Tsol18 protein in later generations of transformed *A. thaliana* as well as additional efforts in purifying the novel Tsol15 construct provide opportunity for the future.

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7. Appendix

7.1. SDS-Polyacrylamide Gel Electrophoresis Solution (10%, 10 ml)

4.0 ml H_2O
3.3 ml 30% Acrylamide mix (Sigma-Aldrich, St. Louis, Missouri, USA)
2.5 ml 1.5M Tris (ph 8.8)
0.1 ml 10% SDS
0.1 ml 10% ammonium persulfate
4.0 µl TEMED (Pharmacia Biotech, Uppsala, Sweden)

(12%, 10 ml)

3.3 ml H_2O
4.0 ml 30% Acrylamide mix (Sigma-Aldrich, St. Louis, Missouri, USA)
2.5 ml 1.5M Tris (ph 8.8)
0.1 ml 10% SDS
0.1 ml 10% ammonium persulfate
4.0 µl TEMED (Pharmacia Biotech, Uppsala, Sweden)

7.2. SDS-Polyacrylamide Gel Electrophoresis Stacking Solution (3 ml)

2.10 ml H_2O
0.50 ml 30% Acrylamide mix (Sigma-Aldrich, St. Louis, Missouri, USA)
0.38 ml 1.5M Tris (ph 8.8)
0.03 ml 10% SDS
0.03 ml 10% ammonium persulfate
3.00 µl TEMED (Pharmacia Biotech, Uppsala, Sweden)

7.3. SDS-Polyacrylamide Gel Electrophoresis Running Buffer (10X)

8.00 L distilled water
303 g Tris base
1442 g glycine
100 g SDS
* pH adjusted to 8.3 and made up to 10 L with distilled water

7.4. Semi-Dry Transfer Buffer (1000 ml)

5.80 g 48mM Tris base
2.90 g 39mM glycine
0.37 g 0.037% SDS
200 ml 20% methanol
* Made up to 1000 ml with distilled water

7.5. Blotto Blocking Buffer

5 g nonfat dry milk
100 ml PBS (Medicago AB PBS tablets, Uppsala, Sweden)

7.6. SDS-Polyacrylamide Gel Loading Buffer (2X Laemmli Sample Buffer)

4 ml 10% SDS
2 ml glycerol
1.2 ml 1M Tris (pH 6.8)
2.8 ml distilled water
0.01% bromphenol blue as tracking dye