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Development of a Recombinant Attenuated Salmonella Vaccine System for Taenia Solium Cysticercosis in Pigs

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DEVELOPMENT OF A RECOMBINANT ATTENUATED SALMONELLA VACCINE

SYSTEM FOR TAENIA SOLIUM CYSTICERCOSIS IN PIGS

by

MARIA E. SILVA

Under the Direction of Dr. Phang C. Tai

ABSTRACT

Taenia solium is a cestode that has a two-hosts life cycle. The adult tapeworm causes an asymptomatic disease known as taeniasis whereas the larval stage causes a disease called cysticercosis. In humans, the most common localization for the larvae is the central nervous system where it produces the neurological disorder neurocysticerco-sis. Previous works by several research groups around the world have shown that *T. so-lium* is a potentially eradicable parasite. Control programs have included treatment of human and pig populations with antihelmintics in conjunction with health education and are now considering vaccination of naïve piglets.

The potential of a live vector vaccine system to deliver *Taenia solium* Tsol18, a proven protective antigen, to prevent transmission of cysticercosis was investigated. An attenuated strain of *Salmonella enterica* serovar Typhimurium χ9402 was used to develop an oral delivery system. Tsol18 gene was cloned downstream from the β-lactamase signal sequence in a multicopy *asd* $^+$ plasmid vector pYA3620 to yield plasmid pYA3620/Tsol18 and then transformed into the vaccine strain. The recombinant attenuated salmonella vaccine construct was stable for 50 generations and expressed rTsol18. Immunization of mice either with one or two doses of 10^9 CFU of the recombinant vaccine strain carrying plasmid pYA3620/Tsol18 elicited specific antibody response to *Salmonella* self antigens and to rTsol18. Moreover, oral immunization of piglets with 10^{12} CFU of the vaccine construction significantly reduced the numbers of viable cysts after challenged.

The development of a quantitative assay to detect specific antibodies against Tsol18 is also presented here. The Falcon assay screening test –enzyme linked immunoabsorbant assay (FAST-ELISA) format was used to develop a quantitative antibody detection assay. We have cloned, expressed and purified rTsol18. With purified porcine IgGs we constructed a standard curve that can be used to quantify the immune response. Our Fast-ELISA was able to follow the kinetics of the immune response in vaccinated pigs from an experimental trial. The data we present here provides the basis for a safe, affordable and easy vaccine delivery system that can be used as an adjunct in control programs.

INDEX WORDS: *Taenia solium*, Cysticercosis, *Salmonella*, Vaccine, Tsol18, Fast-ELISA.

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LIST OF ABREVIATIONS

Ab   Antibody

Ag   Antigen

asd   β-aspartate semi-aldehyde dehydrogenase

BSA   Bovine serum albumin

CFU   Colony forming unit

CSF   Cerebrospinal fluid

CNS   Central Nervous System.

Cox1   Cytochrome C oxidase subunit 1

CT   Computarized Axial Tomography

CV   Coefficient of variance

Cytb   Cytochrome b

DAB   3,3' diaminobenzidine

DAP   Diaminopimelic acid

DNA   Deoxyribonucleic acid

EDTA   Ethylenediaminetetracetic acid

EITB   Enzyme-linked immunotransfer blot

ELISA   Enzyme-linked immunosorbent assay

FAO   Food and Agriculture Organization

FnIII   Fibronectin type III

gDNA   Genomic Deoxyribonucleic acid

GST   Glutathione s-transferase

HCl   Hydrochloric acid

Ig	Immunoglobulin
IgG	Immunoglobulin G
Igs	Immunoglobulins
IPTG	Isopropil-β-D-thiogalactoside
KCl	Potassium Chloride
kDa	Kilodaltons
kg	kilograms
LPS	Lypopolysacharide
mg	milligrams
ml	milliliter
MRI	Magnetic Resonance Imaging
mtDNA	Mitochondrial Deoxyribonucleic acid
NaCl	Sodium chloride
Na_2HPO_4	Sodium phosphate
NaH_2PO_4	Sodium dyhidrogen phosphate
NCC	Neurocysticercosis
OMS	Organizacion Mundial de la Salud
OPS	Organización Panamericana de la Salud
PBS	Phosphate saline buffered
PCR	Polymerase Chain Reaction
PrIgG	Porcine immunoglobulin G
RASV	Recombinant attenuated *Salmonella* vaccine
RFLP	Restriction Fragment Length Polymorphism
RT	Room temperature

SE	Standard error
SDS	Sodium dodecil sul;fate
SDS-PAGE	Sodium dodecil sulfatepolyacrilamyde gel
SOMP	*Salmonella* outer membrane protein
TMB-sure blue	3,3',5,5' Tetramethylbenzidine
UCLA	University of California, Los Angeles
US	United States
USA	United States of America
USC	University of Southern California
UTR	Untraslated region
WHO	World Health Organization

I.   General Introduction

Taenia solium/Cysticercosis is a highly endemic parasitic disease in developing countries where transmission is associated with pigs raised in close contact with humans and with poor hygiene and sanitary condition (Garcia and Del Brutto, 2005; Flisser et al., 2006). Epidemiological studies carried out during the last two decades have demonstrated that cysticercosis is highly prevalent in Cameroon, Tanzania, Zambia, Madagascar, India, Nepal, parts of China, Vietnam, Papua New Guinea (Irian Jaya), Mexico, Ecuador, Colombia and Peru among others (Flisser et al., 2003; Carabin et al., 2006).

Although cysticercosis is considered an imported disease in developed countries, immigration, immigrant domestic workers, and international travel have increased the prevalence of the disease in these non-endemic areas. For example, in the United States cysticercosis has emerged as a common cause of seizures among immigrants from Mexico and other endemic area (Schantz et al., 1992; Schantz, 2006). Infections with the pork tapeworm are still common in developing countries and constitute a public health problem because they produce thousands of infective eggs for humans and pigs causing clinical diseases known as neurocysticercosis and cysticercosis. Immigrants from endemic countries carrying adult tapeworms have been shown to spread the disease to industrialized countries in recent years (Schantz et al., 1993).

I.1 *Taenia solium* life cycle

Taenia solium has a complex two-host life cycle. Humans are the only natural definitive host harboring the adult tapeworm whereas pigs and humans can both act as intermediate hosts harboring the metacestodes (Figure I.1). After ingestion of undercooked pork meat infected with cysticerci, the larva evaginate, attaches to the duodenal wall and develop into a tapeworm (Garcia et al., 2003a). The tapeworm grows and proliferates producing hundred of hermaphroditic proglottids and undergo differentiation to form gravid proglottids full of infective eggs (Naquira, 1999) .
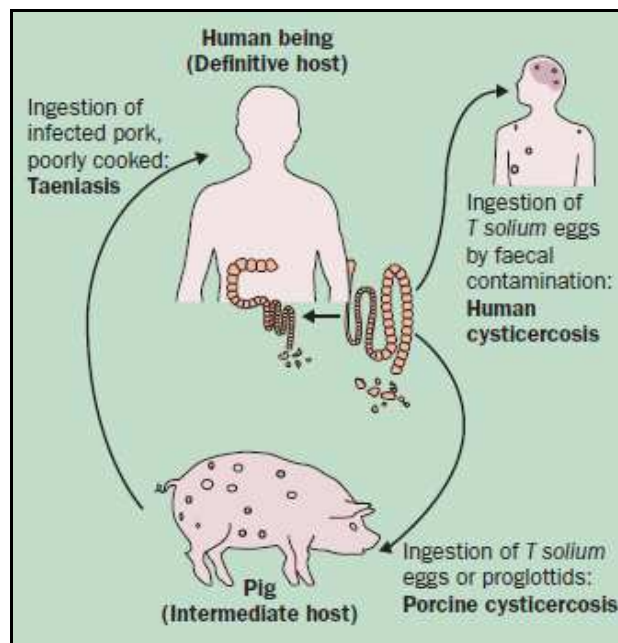
Figure I.1 *Taenia solium* life cycle *

* Source (Garcia et al., 2003a)

The eggs and distal gravid proglottids are eliminated in the feces of a human infected with the adult tapeworm and can contaminate the environment. Pigs became infected with cysticerci after they eat human feces with *Taenia* eggs. After ingestion, the oncosphere hatches and releases the embryo which penetrates the intestinal wall reaching the general blood circulation, and presumably, are transported to different tissues, specially muscle were they develop into cysts (Flisser, 1986). Each egg with an oncosphere can develop into a larva, or metacestodes, a process which takes about 8 weeks (Lapage, 1974).

Humans can develop the cysts after accidental ingestion of *T. solium* eggs in contaminated food. Contact with tapeworm-infected individuals is believed to be the most probable mechanism for infection along with poor hygienic habits allowing for the contamination of hands by eggs, and thus the likelihood of contaminating food for the family group (Flisser, 1986; Garcia et al., 2003a) . Also, eggs that contaminate short-term vegetables and/or water for consumption can also be ingested, particularly in areas with poor environmental sanitation and open air defecation. It has also been claimed; that intestinal retroperistaltic movements induced in *T. solium* carriers by any cause, would allow the gravid proglottids or eggs to reach the stomach, and here, the action of the gastric fluids release the hexacanth embryos, thus producing an internal autoinfection (Loo and Braude, 1982) .

I.2   History and evolution

Phylogenetic analysis of *Taenia* species have shown that species of *Taenia* that infects humans did not form a clade and may represent two independent shifts. The phylogenetic evidence also showed that African hominids that preyed on antelopes and other bovids were exposed to *Taenia* infection. These *Taenia* species that first infected humans were using hyanids, canids and felids as definitive hosts and bovids as intermediate ones. Therefore, it can be assumed that *Taenia* species became associated with humans 10,000 years ago wen domestication of the intermediate host occurred (Hoberg et al., 2001) . Furthermore, immigration of human clans from Africa to Eurasia brought the *Taenia* ancestor along with them (Hoberg et al., 2001).

Species of *Taenia* were among the earliest recognized parasites in human written records. The earliest reference to tapeworms was found in the work of ancient Egyptians (2000 BC). A study published in 2006 described a case of cysticercosis on a Egyptian mummy dating to the Ptolemaic period (Bruschi et al., 2006). *Taenia solium* was apparently well known by the Greeks. The description of "measly pork" in the History of Animals written by Aristotle (384-322 BC) showed that the infection of pork with tapeworm was known to ancient Greeks. However, there is no evidence to suggest that the Greek physicians knew that some humans harbored cysts in the brain or suffer from epileptic episodes that were associated with them (Cox, 2002; Wadia, 2002).

During the colonial era the European isolates were introduced to Latin America along with pigs (Nakao et al., 2002; Ito et al., 2003). Cysticercosis was described by Johannes Udalric Rumler in 1555; however, the connection between tapeworms and cysticercosis had not been recognized at that time (Wadia, 2002). Before the relationship between *Taenia* species and their larval stages were completely understood the cysticerci were described as independent species with their own scientific names. It was not until 1784 when Johann Goeze recognized that the cysticerci were the larval stages of tapeworm (Cox, 2002).

Scientific studies of *Taenia* species can be trace back to 17th century when Edward Tyson recognized for the first time the tapeworm head (scolex). Even though it was clear now that there were significant morphological differences between various species of tapeworms, it was not until the middle of the 19th century that the differences between *T. solium* and *T. saginata* were first recognized by Kuchenmeister. It was during the same century that, Kuchenmeister performed an experiment in which he fed undercooked pork containing cysticerci of *T.solium* to humans awaiting execution in a prison, and after they had been executed, he recovered the developing and adult tapeworms in their intestines. It was also established around this time that cysticercosis was caused by ingestion of *T. solium* eggs (Cox, 2002).

Recently, phylogenetic polymorphism was demonstrated among 13 *T. solium* isolates from different regions around the world. PCR amplification of 2 mitochondrial genes: cytochrome *c* oxidase subunit 1 (Cox1) and cytochrome *b* (Cyt*b*) showed that isolated from China, Irian Jaya, India and Thailand formed a single cluster whereas the

isolated strains from Peru, Mexico, Ecuador, Bolivia and Brazil together with those from Tanzania, Mozambique and Cameroon formed a different cluster. These polymorphism probed that *T. solium* that was introduced into Latin America had a different origin from the one spread in Asia (Nakao et al., 2002).

I.3   *Taenia solium* morphology

I.3.1   Adult tapeworm

Taenia solium is a flat, segmented hermaphrodite parasite that can grow up to eight meters in length in the small intestine of the human host. Morphologically, 3 portions can be recognized in the adult tapeworm: the head, the neck and the body or strobile. The head, also known as scolex (Figure I.2) is a 1 millimeter structure that has a pear shape bearing four muscular suckers for fixation and has a small terminal cone known as rostelum. At the base of the rostelum, there is a double row of hooks which can number from 22 to 36 (Naquira, 1999).
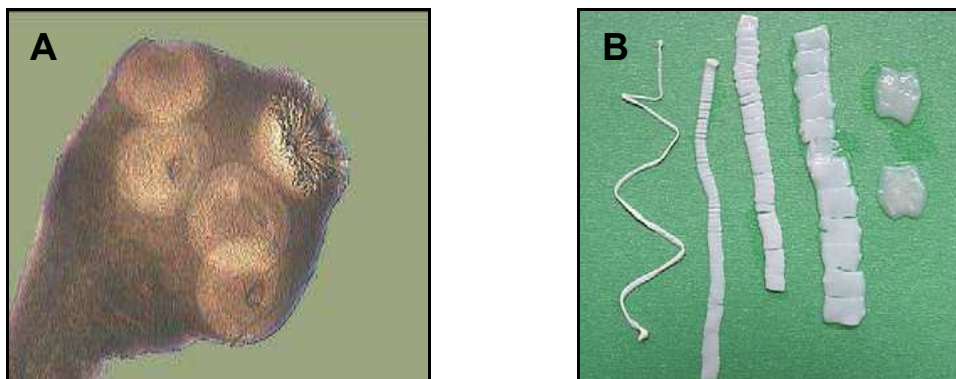
Figure I.2 *Taenia solium* adult tapeworm *

A: *Taenia solium* scolex; **B**: Strobile of adult tapeworm. The size and form of the proglottids change as they mature (Garcia and Del Brutto, 2000).
* Source (Garcia et al., 2003).

The neck is a poorly differentiated structure, made of germinative tissue with high mitotic activity located immediately posterior to the scolex, which measures 5 to 10 millimeters long. It is the portion where the formation of immature proglottids starts (Naquira, 1999).

The body or strobile is the longest portion of the parasite and is made up of a series of segments known as proglottids. Proglottids are covered by a tegumentary surface that constitutes the absorption surface through which the worm takes up metabolites from the host (Willms, 2008). The genital pores are located on each side of each proglottid. The strobile has immature, mature and gravid proglottids. Immature proglottids are undifferentiated and located next to the neck. Mature proglottids are complete reproductive units with a complete set of male and female reproductive organs that may undergo

self or cross fertilization. Gravid proglottids are located in the posterior part of the body of the parasite and have a highly branched uterus filled with eggs. Gravid proglottids are cut off from the tapeworm body and are released with the host feces (Naquira, 1999).

I.3.2 Oncosphere

T. solium uterus contains multiple spherical eggs that contain the hexacanth embryo also known as oncosphere (Figure I.3). *T. solium* eggs are composed of a cho-rionic membrane, an embryophore, an embryophoral membrane, and two oncosphere membranes that surround the embryo. *T. solium* eggs are very resistant to common dis-infectants and environmental conditions. They can survive in humid ground for months (Bowman, 1999).

Figure I.3 *Taenia solium* oncosphere

I.3.3 Cysticercus

The cysticercus is the larval stage of *T. solium*, called *Cysticercus cellulosae* by Rudolphi (1803) given its predilection for conjunctive tissue. Morphologically, the cysticercus is a translucent vesicle full of liquid, measuring between 0.5 and 2 cm in diameter with an invaginated scolex. The cysticercus vesicle is surrounded by a thin layer of fibrous tissues that separates it from the surrounding tissue (Lapage, 1974) (Figure I.4). Sometimes, depending on the location of the cyst, this morphology can vary to include irregular forms such as racemose. Racemose cysticercus looks like a large lobulated vesicle, similar to a bunch of grapes. It may measure up to 10 cm of diameter and contain several milliliters of fluid and is generally observed in the ventricular cavities and in the cisterns located at the base of the cranium (OMS/OPS, 1993).

The histological structure of the bladder wall consists of three layers and differs from the parenchymatous portion containing the invaginated scolex which is sealed inside the bladder cavity. The cysts parenchyma is composed of a complicated system of cells, slits, fibers and channels that permit the invagination of the scolex. The invaginated scolex forms a spiral canal; the suckers are present at its end from which the rostelum with hooks arises (Willms, 2008).

In the initial phase of the cysticercus growth, the invaginated scolex and spiral canal leading to it are developed in the parenchymatous portion of the elongated larval body. The formation of the invaginated scolex and the development of the spiral canal change the initially poorly differentiated bladder. The cyst attains its final shape after a

secondary developmental phase during which the anterior parenchymatous portion of the larval body becomes completely enveloped by the posterior part through a secondary growth of the bladder, giving rise to a typical cyst. In vitro, when a viable cysticercus is exposed to bile, the larvae evaginate a process whereby the scolex emerges from the vesicle. In vivo the evagination process releases the scolex into the duodenum where it attaches into the wall of the definitive host (Willms, 2008).
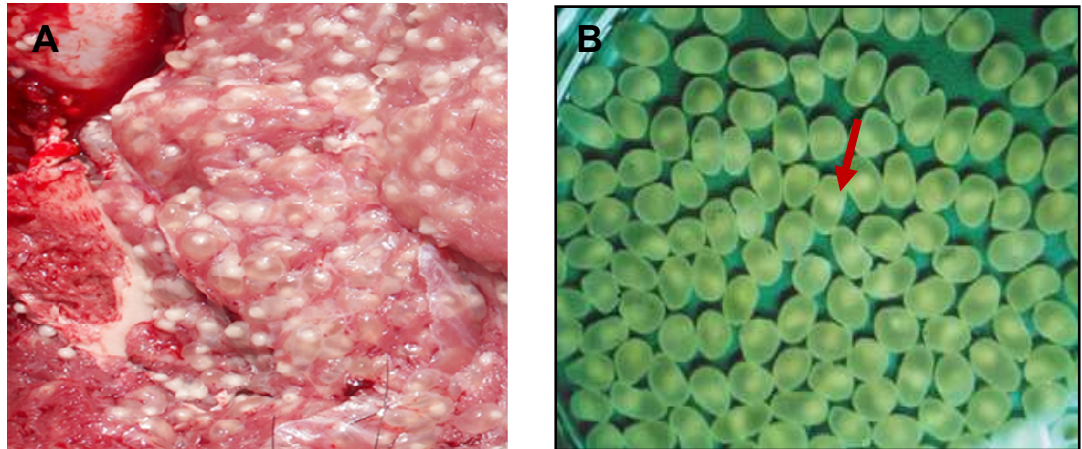
Figure I.4 *Cysticercus cellulosae*

A: *Cysticercus cellulosae* as seen in a heavily infected pig; **B**: Excised in a petri dish the white dot pointed with an arrow corresponds to the scolex (Garcia and Del Brutto, 2000).

I.4   Clinical importance of Taeniasis and cysticercosis

I.4.1   Taeniasis

Taeniasis (infection by an adult tapeworm) occurs only in the human host. Adult *T. solium* carriers present few or no symptom, although some patients presented abdominal pain, distension, diarrhea and nausea (OMS/OPS, 1993). The association between the presences of the parasite and weight loss of the patients has not been demonstrated. Thus, the general condition of the individual is not affected. Some people eliminate pieces of strobile in their feces. Identification of *T .solium* infections becomes important because of the risk of cysticercosis not only for the tapeworm carrier but also for its surrounding environment (Garcia et al., 2003a). Frequency of autoinfection in tapeworm carriers is unknown, however; approximately 25% of cysticercosis patients harbor also the tapeworm (Dixon, 1961).

I.4.2   Cysticercosis

In humans, the clinical importance of the infection appears when the metacestode develops in the central nervous system producing neurological disorders, or NCC. Although there is an active infection, the disease typically remains unrecognized during the early stages unless the cyst obstructs cerebral spinal fluid (CSF) circulation (Garcia and Del Brutto, 2000; Garcia and Del Brutto, 2005). After variable times, the cysts start to degenerate and produce an immune mediated inflammation, which results in clinical manifestation of the disease. NCC symptoms are related to number, size and localiza-

tion of the parasites as well as in the severity of the host's immune response. Late onset of epileptic seizures is the commonest and sometimes the only manifestation of NCC. Seizures occur in 50 to 80% of patients with cysts in the parenchyma or with calcifications. NCC patients can also develop intracranial hypertension and hydrocephalus as a consequence of the presence of cysts in the cerebral ventricles or basal cisterns of the brain (Garcia et al., 2003a). Even though the central nervous system is the most relevant clinical localization for the cysticerci they can also be found in muscle and subcutaneous tissue.
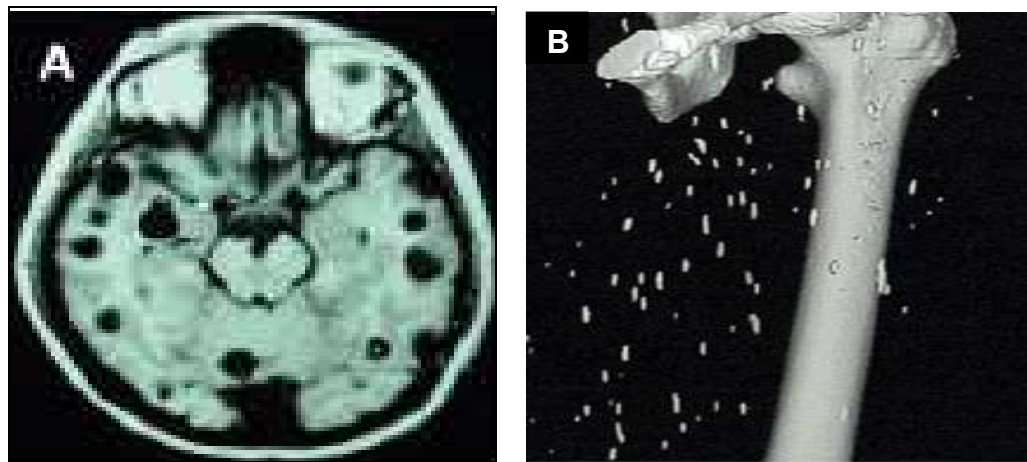
Figure I.5 Human cysticercosis *

A: Human neurocysticercosis, MRI caverns in the brain indicate the presence of viable cysts; **B**: Cysticercosis muscular localization calcified cysts on the CT.

* Source (Garcia et al., 2004)

I.5   Diagnosis and treatment

I.5.1   *Taeniasis*

Diagnosis of taeniasis in human faces two major problems for diagnostic. The first problem is the lack of sensitivity of the microscopic stool examination and the second one is the morphological similarities between *T. solium* and *T. saginata* eggs. Microscopic visualization of *Taenia* eggs present in stool samples was for many years the only available diagnostic method (Garcia et al., 2003a). The detection in stool samples of tapeworm metabolic products with antigenic capabilities has been used to prepare specific polyclonal antibodies that allow the detection of coproantigens by ELISA. Coproantigen detection has a specificity and sensitivity of 100% and 98% respectively (Allan et al., 1990; Allan et al., 1992).

In 1999, Wilkins et al described *T. solium* specific antigens to detect serum antibodies using the western blot assay (Wilkins et al., 1999). The western blot showed a 95% sensitivity and 100% specificity (Levine et al., 2004). No cross reaction with samples from patients with *T. saginata* or other cestode infections were reported. However, one samples from a neurocysticercosis patient without taeniasis tested positive (Allan et al., 2003). Diagnosis of taeniasis by serological examination has some advantages over stool sample examination methods. However, residual antibodies from previous infections or exposure might result in a false positive diagnostic (Allan et al., 2003; Ito and Craig, 2003).

During the last two decades highly specific molecular methods to detect *T. solium* DNA in stool such as PCR and RFLP have been developed (Mayta et al., 2000; Nunes et al., 2003; Nunes et al., 2005; Yamasaki et al., 2005; Nunes et al., 2006; Dias et al., 2007; Mayta et al., 2008) but none of them have been validated under field conditions yet.

There are two available drugs to treat adult *T. solium* infections: praziquantel and niclosamide. Niclosamide is the best choice for adult *T. solium* infections since it is not absorbed from the intestinal lumen. Niclosamide is used as a single dose of 2 gram given orally. On the other hand, there is a small risk with praziquantel that asymptomatic viable cysts in the brain could be affected by the drug and develop neurological symptoms like seizures in the patient. The recommended dose of praziquantel is 5 – 10 mg/kg given orally on a single dose and under medical supervision (Garcia et al., 2003a).

I.5.2 Cysticercosis

Clinical manifestations of cysticercosis depend on the affected organ or tissue. Neurocysticercosis and ophthalmic cysticercosis are often associated with substantial morbidity (Garcia and Del Brutto, 2005). Immunodiagnostic methods to detect either circulating antigens or antibodies in serum, cerebrospinal fluid and urine have been described (Tsang et al., 1989; Brandt et al., 1992).

The enzyme-linked immunotransfer blot (EITB) for circulating antibody detection is the WHO recommended screening test for cysticercosis. This assay developed by Tsang in 1989, was originally reported to have 98% sensitivity and 100% specificity (Tsang et al., 1989) and performs better in field condition than conventional ELISAs (Proano-Narvaez et al., 2002). The EITB requires expensive reagents advanced equipment and highly trained technicians to perform the assay. However, when available the immunoblot is the best diagnostic tool (Garcia et al., 2003a).

Antigen detection assay has lower sensitivity and specificity than the EITB but can detect live parasites. Some assay based in monoclonal antibodies performed well on cerebrospinal fluid however there is limited evidence on its sensitivity and specificity when the test is performed in serum samples (Garcia et al., 1998; Garcia et al., 2000). Since number, localization size and stage of the cysts are important for the physician in order to treat the infected patient neuroimaging such us MRI and CT become the most important tools for cysticercosis diagnostic in humans.

Until 1978, the only treatment for neurocysticercosis was surgery. Years later, Praziquantel became the first specific drug to be used in cysticercosis treatment (Sotelo et al., 1984; Sotelo, 1997). Later, albendazole became available and represented a more effective, cheaper and convenient way to treat patients (Escobedo et al., 1987; Sotelo et al., 1988a; Sotelo et al., 1988b; Escobedo et al., 1989). Cysticercosis outside the central nervous system is considered a disorder that does not need to be treated. Porcine cysticercosis is treated using a single dose of 30 mg/Kg oxfendazole given orally (Gonzales et al., 1996).

I.6   Epidemiology

T. solium/Cysticercosis is a highly endemic parasitic disease in developing coun-tries where transmission is associated with pigs raised in close contact with humans, and with poor hygiene and sanitary conditions (Chatel et al., 1999; Flisser et al., 2006). Epidemiological studies carried out during the last two decades have demonstrated that cysticercosis is highly prevalent in Cameroon, Tanzania, Zambia, Madagascar, India, Nepal, parts of China, Vietnam, Papua New Guinea (Irian Jaya), Mexico, Ecuador and Peru among others (Phiri et al., 2003; Rajshekhar et al., 2003; Shey-Njila et al., 2003; Wandra et al., 2003; Flisser et al., 2006; Wandra et al., 2006). Although cysticercosis is considered an imported disease in developed countries, immigrant domestic workers and international travel have increased the prevalence of the disease in these non-endemic areas (Figure I.6). For example, in the United States cysticercosis has emerged as a common cause of seizures among immigrants from Mexico and other endemic areas (Schantz et al., 1994; Schantz, 2006). In the US it is estimated that over 1000 people per year are diagnosed with cysticercosis, the majority of those immigrants from endemic countries. Retrospective studies performed by the Health Services in the State of California, USA indicated cysticercosis as the cause of death of 124 persons between 1989 and 2000. In addition, a study carried out at UCLA Medical Center revealed an in-crease of 14% in NCC admission during the period from 1994 to 1998. Another retros-pective study showed that 10% of the neurology and neurosurgery admission in Los An-geles County USC Medical Center were due to NCC during 1994 to 1999. Although the majority of cases of NCC as well as tapeworm carriers in the US have been diagnosed among immigrants from endemic areas; immigrants become potential foci for disease

transmission to other family members and non-immigrant contacts (Sorvillo et al., 2004; DeGiorgio et al., 2005a; DeGiorgio et al., 2005b).

NCC affects older children and adults, therefore, serious economic impact due to disability are expected (Garcia and Del Brutto, 2005). Studies performed in Mexico and published in 1989, (Flisser et al.) estimated a cost of US $15 million per year only in hospital admissions of new cases of NCC (Flisser, 1988). In addition, porcine cysticercosis causes the loss of US $43 million per year as a consequence of the confiscation and destruction of contaminated carcasses. In Eastern Cape Province (South Africa) the cost of *T. solium* cysticercosis has been estimated to vary from US $18.6 to US $34.2 million per year with US $5 million corresponding to the cost for the agricultural sector (Carabin et al., 2006). For Latin America, porcine cysticercosis accounts for an estimated economic loss of US $64 million (Schantz, 2006).
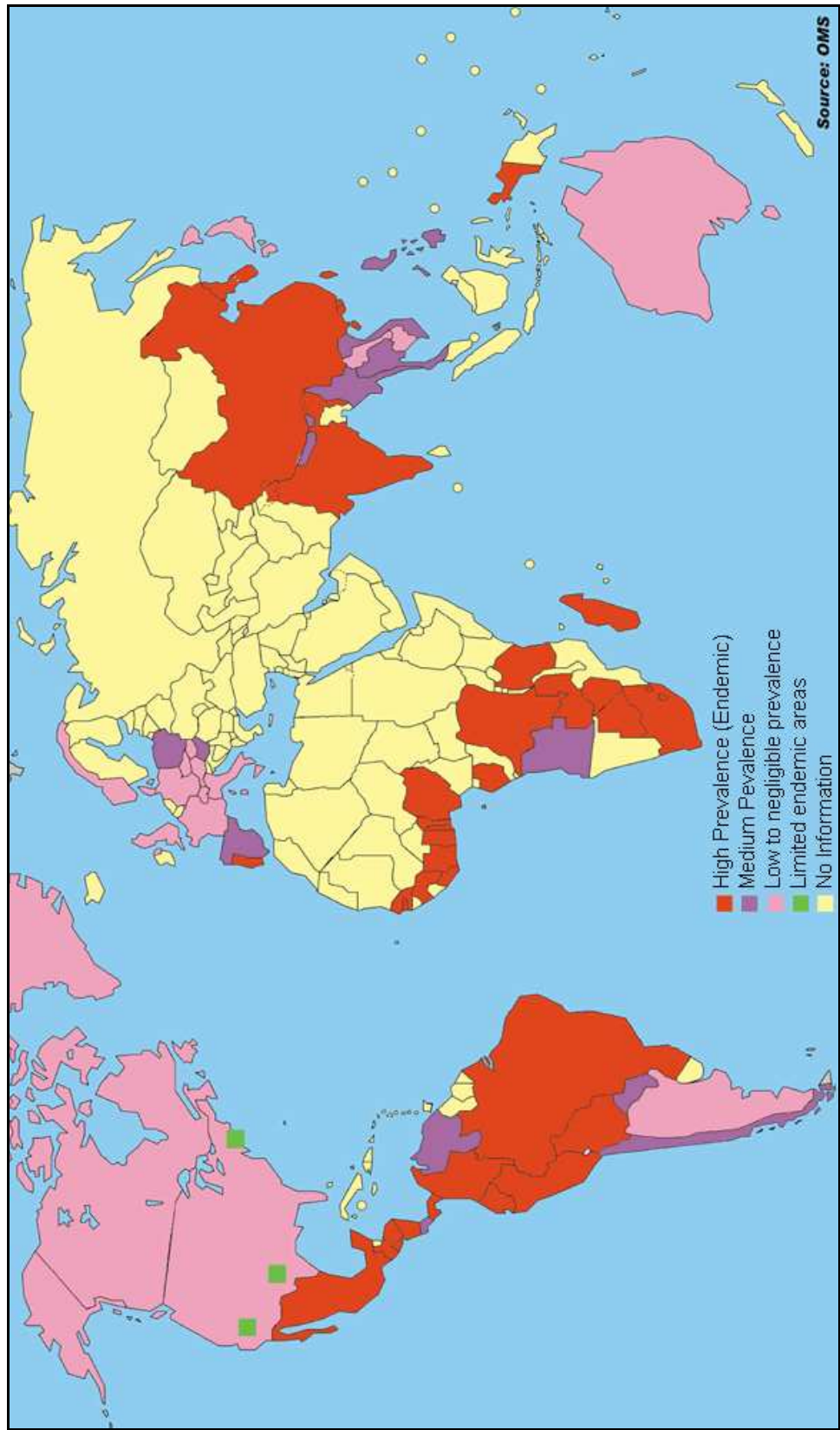
Figure I.6 Distribution of human cysticercosis*

*Source WHO (2002)

I.7   Control

In an effort to interrupt the transmission, several control measures such as mass treatment of pigs, identification and treatment of tapeworm carriers, education and sanitation have been implemented and tested (Gonzalez et al., 2003; Sarti and Rajshekhar, 2003). Mass treatment with praziquantel and niclosamide to eliminate tapeworms from human carriers have shown reduction in prevalence of the disease (Allan et al., 1997; Sarti et al., 2000). It has also been shown that improvement of pig husbandry, and sanitation to deter access by pigs to human feces reduced the prevalence of cysticercosis in rural areas (Sarti et al., 1994).

Although a number of intervention trials based on health education and improved sanitation have demonstrated that transmission of *T. solium* can be inhibited temporarily (Flisser et al., 2003), it has become clear that an effective elimination of *T. solium* will require sustained pressure on the parasite during both stages of its development (Schantz, 2006). Currently, no control intervention has achieved definitive interruption of the parasite life cycle, and recrudescence occurs as a consequence of tapeworm reintroduction (Schantz et al., 1993; Garcia et al., 2003a; Sarti and Rajshekhar, 2003). One measure being considered to help prevent disease reintroduction is vaccination of naïve pigs (Lightowlers, 1999). Vaccination of the intermediate host will remove the source of human infection and therefore the *T. solium* life cycle would be interrupted (Flisser et al., 1979; Lightowlers, 1999; Lightowlers, 2003).

I.7.1 Vaccination

Immune response generated by *Taenia* infection is basically antibody mediated. During the sixties, Gemmel and Blundell reported that the immune response against *Taenia* infection is composed of an early and a late immune response (Blundell et al., 1968; Gemmell et al., 1968; Blundell et al., 1969). The early immune response may act at the intestinal level, while the late immune response does it at the site of metacestode development. In addition, he also described that antibodies generated during the early stage of infection are likely to be protective and that these antibodies were likely to be generated against metabolic products of embryos undergoing metamorphosis (Gemmell and Lawson, 1989; Lightowlers, 1990). Based on these observations, studies were conducted in order to identify each oncosphere antigen. In the particular case of *T. ovis*, studies identified several antigenically distinct oncosphere antigens. Three of these antigens, To45W, To16 and To18 were cloned, expressed in *E. coli,* and shown to be protective against challenge (Harrison et al., 1993; Harrison et al., 1996). *T. ovis* oncosphere work lead to the identification and cloning of homologs of these three antigens: from *T. saginata* and *T. solium* as well as from *Echinococcus granulosus* (Johnson et al., 1989). Some of these antigens have been tested and found to be protective for the intermediate host (Lightowlers et al., 2003; Flisser et al., 2004; Gonzalez et al., 2005).

For *T. solium*, homologs to To45W and To18 known as Tsol45 and Tsol18 have been cloned and tested in 5 different trials (Table I.1 and Table I.2). The first vaccination trial performed in Mexico showed 100% protection in those animals immunized with 2 doses of 200 µg of recombinant GST-Tsol18 fusion protein; whereas, vaccination with

Tsol45 showed no protection. On a second trial also performed in Mexico, 4 doses of recombinant Tsol45 were necessary to obtain protection levels similar to those obtained with Tsol18 (Flisser et al., 2004). The third trial performed in Cameroon, tested only Tsol18 and showed 100% of protection. The fourth and fifth trials were performed in Peru. Both of them showed high levels of protection in animals immunized with Tsol18 (Gonzalez et al., 2005).

Table I.1 Induction of protection against *Taenia solium* cysticercosis in pigs afforded by vaccination

Vaccine	Location	Dose	No. Animals	Mean No. of cysts per animal	Protection (%)[*]
GST (control)	Peru	2 doses	5	1599.2	0
GST/MBP	Peru	2 doses	5	1669.4	0
GST/Tsol 18	Peru	2 doses	8	0.5	99.9[**]
GST/MBP/ Tsol45	Peru	2 doses	8	22.3	98.6[**]
GST (control)	Mexico	2 doses	5	256.8	0
GST/Tsol18	Mexico	2 doses	5	0	100
GST/Tsol45	Mexico	2 doses	5	136.2	46.96
GST/Tsol18-GST/Tsol45	Mexico	2 doses	5	13.6	94.7[**]
GST (control)	Mexico	4 doses	12	41.8	0
GST/Tsol18	Mexico	3 doses	5	0.2	99.5[**]
GST/Tsol45	Mexico	4 doses	5	1.2	97.1[**]
None	Cameroon	2 doses	5	30	0
GST/Tsol18	Cameroon	2 doses	5	0	100[***]

* Protection was calculated as the percent reduction in the mean number of cysticerci in each group in comparison with the mean in the respective control

Data from (Flisser et al., 2004; Gonzalez et al., 2005).

Table I.2 Protection against cysticercosis in vaccinated pigs

Vaccine	Location	Dose	No. of animals	Mean of No. of live cysts per animal	Protection (%)
6his (control)	Peru	2 doses	5	241.6	0
GST (control)	Peru	2 doses	7	388.3	0
6his/Tsol 18	Peru	2 doses	5	60	75.2[*]
GST/Tsol18	Peru	2 doses	8	3.75	99[*]

* Protection (%) was calculated as a percent of reduction in the mean number of live cysts in each group compared with the mean in the respective control.

Source: Hancock unpublished data

Tsol18 is a homolog to the 18 kDa protective oncosphere antigen from *T. ovis* (To18). Tsol18 gene has 3 exons and 2 introns. Two small exons encode for the amino and the carboxy terminal domain. A single larger exon encodes the protein. In addition, Tsol18 also has an intron in the 3'untranslated region (UTR) (Gauci et al., 1998; Gauci and Lightowlers, 2003). Analysis of the predicted protein sequence encoded by the first exon shows the presence of a hydrophobic domain at the N-terminus which is likely to be a secretory signal. The second exon encodes for a fibronectin type III domain (FnIII) which is conserved among all the protective *Taenia* oncosphere antigens characterize thus far (Gauci and Lightowlers, 2003; Gauci et al., 2006). FnIII domains are highly conserved and have 100 aminoacids arranged into a β sandwich fold composed by three β strands in one sheet and four in the other one. Tsol18 has a single copy of this domain. Tsol18 mature protein (Genebank accession N° AF017788) codes for 113 amino acids which includes a group of 14 aminoacids (from Threonine at position 3 to phenylalanine at position 26) at the beginning of the sequence forming a hydrophobic area (Figure I.7). Tsol18 has a molecular weight of 12.8 kDa; a putative glycosylation site, and is specifically expressed during the oncosphere stage (Gauci et al., 2006).
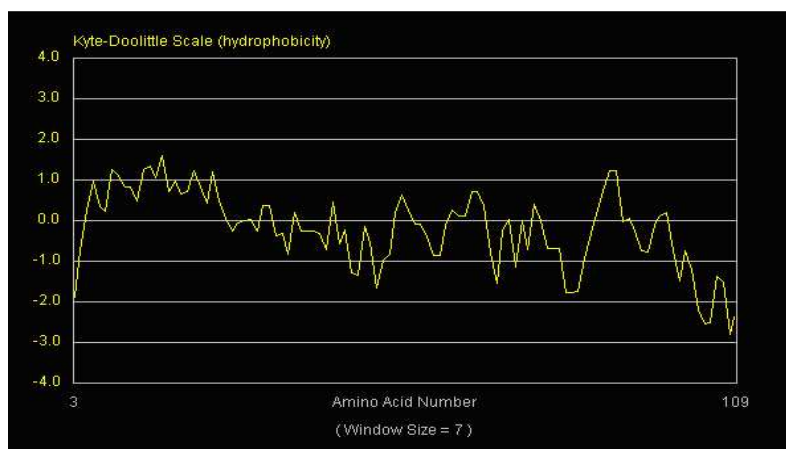
Figure I.7 Mature Tsol18 Hydrophobicity plot (Kyte-Doolittle scale)

I.8   Purpose of the Study

Genetic manipulation of *Salmonella* species has resulted in the loss of their pathogenicity without interfering with their ability to stimulate the immune system. In this regard, attenuated *Salmonella* has the ability to generate a strong mucosal immune response in addition to the humoral and cellular immune responses not only against self antigens, but also against recombinant ones (Dougan et al., 1989). Attenuated and immunogenic strains of *Salmonella* can be genetically engineered to stably express foreign antigens. Recombinant strains used as vaccine delivery systems, usually have a plasmid vector that harbors the sequence for the foreign antigen (Chatfield et al., 1993; Chatfield et al., 1994). These plasmids have to be stably maintained in the bacteria in order to induce an immune response against the recombinant antigen. Stable expression of foreign antigens can be accomplished by integration of the genes specifying for the foreign antigen into the bacteria chromosome. Another approach for stable and high level expression is the balanced-lethal host-vector (Curtiss III, 1989; Curtiss et al., 1989b; Curtiss et al., 1990; Curtiss et al., 1994). The stable balanced-lethal host-vector uses an attenuated strain of *Salmonella* harboring a mutation in the β-aspartate semi-aldehyde dehydrogenase (*asd*) which imposes a requirement for diaminopimelic acid (DAP), and a plasmid vector that complemented that defect in which the recombinant protein is cloned. Thus, loss of the plasmid resulted in cell death and lysis. Several foreign antigens from pathogens closely related to *Salmonella* and also from viruses, or parasites have been expressed in recombinant avirulent *Salmonella* and tested for safety and efficacy against challenge in animal models (Koji Nakayama, 1988). Good immune responses were observed when the foreign antigen is retained in the cytoplasm of the re-

combinant *Salmonella* as well as when foreign antigens are localized to the periplasmic space, or to the outer membrane (Khan et al., 1994; Chabalgoity et al., 1997; Villarreal-Ramos et al., 1998; Kang et al., 2002)**.**

Recently, Lightowlers and others have shown that the oncosphere antigen known as Tsol18 can confer protection when it is administrated as a recombinant GST fusion protein in 2 doses of 200 µg each or as a His-tagged protein (Flisser et al., 2004; Gonzalez et al., 2005; Hancock, unpublished data). Although Tsol18 has been proved to be a good vaccine candidate, the production of a recombinant protein for vaccine use is expensive, and therefore impractical for large-scale use. The use of recombinant attenuated *Salmonella* strains to deliver the vaccine candidate will not only reduce the cost of vaccine production but also will reduce the numbers of vaccine doses needed per animal, since one dose will be enough to protect pigs from infection during its productive life. Reduction in the number of doses per animal will also facilitate animal management in field. Consequently, the aim of this proposal was to develop and test the efficacy of a safe, effective and inexpensive delivery system for the *T. solium* cysticercosis vaccine candidate Tsol18 that can be added to the already existing control measures and be used in eradication programs in field trials. For this purpose, an attenuated strain of *Salmonella enterica* var typhimurium was used as a vector to deliver the vaccine candidate. The attenuated *Salmonella* (∆*asd*) strains and the plasmids that complement this mutation were available for research in collaboration with Dr. Roy Curtiss III Co-director of the Center for Infectious Diseases and Vaccinology, Biodesign Institute, Arizona State University. The vaccine efficacy was tested by vaccination and challenge of naïve pigs.

Immunity to infection with the larval stage of cestodes parasites is antibody mediated. Early experiments performed in *Taenia ovis* demonstrated that protection can be afforded by the passive transfer of antibody. In order to study and follow the specific immune response generated by the *Salmonella* vaccine construction a serological test in a FAST-ELISA format was developed. The FAST-ELISA was used to quantify and follow the specific immune response in vaccinated animals to establish a correlation between the antibody levels and protection against challenge.

II.   Development and optimization of the FAST-ELISA to detect protective antibodies against cysticercosis in vaccinated pigs

II.1   Introduction

Cysticercosis is a parasitic infection produced by the larval stage of *Taenia solium* (Flisser, 1994). The life cycle of this parasite requires 2 hosts: an intermediate host, the pig, where the cysts develop; and a definitive host, humans, for the adult tapeworm (Flisser, 1986; Garcia and Del Brutto, 2000; Garcia and Del Brutto, 2005). Although pigs act normally as intermediate hosts, humans accidentally infected with *Taenia solium* eggs can also develop cysts. In humans, the most commonly observed location of the larvae is the central nervous system (CNS) where they cause a disease known as neurocysticercosis (Garcia and Gilman, 1995; Garcia et al., 1999). Neurocysticercosis often causes seizures and it is responsible for substantial mortality and morbidity in developing countries (Lightowlers and Gauci, 2001).

Control and eradication programs have shown that transmission of the disease can be interrupted temporarily but recrudescence occurs as a consequence of tapeworm reintroduction and infection of naïve pigs (Garcia et al., 2006). Controlling porcine cysticercosis is vital for eradication and control programs (Garcia et al., 2003b; Gonzalez et al., 2003; Flisser et al., 2006). Studies on immunizing pigs with recombinant vaccine antigens derived from oncospheres showed that one of the vaccine molecules was capable

of inducing protection in 99% of the vaccinated animals against challenge when it is administrated in 2 doses of 200 µg each (Flisser et al., 2004; Gonzalez et al., 2005). Studies on the immune response generated by the recombinant vaccine using a conventional ELISA have been previously reported (Kyngdon et al., 2006a). However, conventional ELISAs cannot be considered truly quantitative assays since they require a dilution titration of the sera and an end point determination (Tsang et al., 1980; Tsang et al., 1983b).

A quantitative, simple and rapid serologic assay based on the Falcon assay screening test (FAST-ELISA) has been previously developed and used to follow the progress of infection and treatment in many diseases (Tsang and Chau, 1987; Levine and Brumley, 1989; Fagbemi and Hillyer, 1990; Hillyer and Soler de Galanes, 1991; Tsang and Wilkins, 1991; Hillyer et al., 1992; Azab et al., 1999). Therefore; the objective of this study was to develop a quantitative FAST-ELISA to detect protection-associated antibodies in pigs vaccinated against cysticercosis.

To develop this quantitative assay we have cloned, sequence, produce and purify the cysticercosis protective antigen rTsol18. This paper describes the FAST-ELISA for detection of specific antibodies against *Taenia solium*-cysticercosis protective antigen rTsol18. In addition, we used the developed test to follow the specific immune response in vaccinated pigs. This test not only meets the criteria of easy operation and quantification but will allow us to monitor vaccinated animals.

II.2 Materials and Methods

II.2.1 FAST – ELISA

The FAST ELISA system consists of sticks molded on to a lid that fits a 96-wells ELISA plate. The antigen is adsorbed on to the sticks, washed and exposed to serum antibodies, enzyme-conjµgated anti-porcine 2nd antibodies, and substrate in 5 minutes incubations. The reaction is stopped by removing the sticks from the substrate. All the steps for the FAST ELISA were performed as previously described (Hancock and Tsang, 1986; Handali et al., 2004).

Briefly, sticks (transferable solid phase screening, catalog #445497; Nunc, Roskilde, Denmark) were sensitized with the antigen diluted in 0.05M Tris/HCl, 0.3M KCl, 2.0 mM ethylenediaminetetracetic acid (EDTA); pH 8.0(sensitization buffer) in 96-well plates (Nunc, catalog #2696200) for 2 hours in an orbital shaker (Titer Plate Shaker; Laboratory-Line Instruments, Melrose Park, IL) at room temperature (RT). Previous studies have shown that absorption of antigens to polystyrene occurs within 2 hours of incubation at RT (Tsang et al., 1980).

After sensitization sticks were washed with 0.15M NaCl in 0.01M Na$_2$HPO$_4$/NaH$_2$PO$_4$; pH 7.2 (PBS), containing 0.3% tween 20 (Sigma Chemical Company) using a garden sprayer. Washes were performed after each incubation step. After each wash, the sticks were rinsed with deionized water. Pig sera were diluted 1:200 in PBS; 0.3% tween 20; 5% nonfat dry milk and tested in triplicates. Samples with activity higher than the maximum point in the standard curve were further diluted and retested

until their activity fell within the standard curve. In homemade affinity purified goat anti porcine IgG (heavy and light chain), horseradish peroxidase labeled and diluted, 1:3000 was used as conjugate (Tsang et al., 1995). 3, 3', 5, 5'Tetramethylbenzidine; TMB-Sure blue (Kirkegaard and Perry Laboratories) was used as substrate. The substrate solution was dispensed into the plate and the sticks were incubated again as in the previous steps for 5 minutes. The reaction was stopped by removing the sticks from the substrate. The absorbance was measured at 630 nm in a ThermoMax microtitration plate reader (Molecular Devices, Sunnyvale, CA). The concentration of each sample was derived from the standard curve and expressed in micrograms/ml.

II.2.2   Standard curve preparation

The immunoglobulin G (IgG) standard curve was prepared as described by Scheel (Scheel et al., 2006). Briefly, normal porcine immunoglobulin G (PrIgG) was precipitated using ammonium sulfate $(NH_4)_2SO_4$; purified by ion exchange chromatography using a Gamma G Hi-Trap (Amersham Biosciences) column and quantified by Bradford protein assay. Purified PrIgGs were stored in liquid nitrogen until use. In order to prepare the standard curve, PrIgGs were diluted in PBS; pH 7.2, 0.1% sodium azide, 0.001% bovine serum albumin (BSA) fraction V, protease free (Catalog No:3116999001, ROCHE) to a working dilution of 100 µg/ml. A pilot curve of 11 points was constructed in the following concentrations (µg/ml): 12.5, 6.25, 3.18, 1.6, 0.8, 0.4, 0.2, 0.1, 0.05, 0.025 and 0. The diluted standards were stored at -4° C. The standards were assigned with reactivity values that corresponded with their concentration. The standard curve was tested in triplicates.

II.2.3 Optimization of assay reagents

In the FAST-ELISA format all the reagents with the exception of the one being measured need to be added in excess. To determine the amount of antigen to be considered in excess the antigen titration was performed. Briefly, decreasing 2-fold dilutions of Tsol18 in sensitization buffer (from 10µg/ml to 0µg/ml) were coated onto the beads in triplicates. In order to titrate the antigen, the remaining reagents used for the ELISA were added in excess. Sera from a hiperimmunized goat diluted 1:4 was used as a source of antibodies. Titration of the conjugate was performed by assaying several conjugate dilutions. Antigen and antibody in excess were used for the assay. A conjugate dilution of 1:3000 in PBS- 0.3% tween 20 was determined to be in excess.

II.2.4 Antibody titration

An antibody titration was performed using a pool of serum from vaccinated pigs to determine the dynamic range of the assay. For the titration, plates were sensitized with 2µg/ml of purified Tsol18 antigen (this is antigen in excess). Serial 2-fold dilutions of the positive pool sera in PBS - 0.3% tween 20/ 5% non fat milk were assayed in triplicates in FAST-ELISA. A serial 2-fold dilution of normal porcine sera was assayed in the same plate as a control. Signal to noise ratio was calculated for each sera dilution.

II.2.5   Sera samples

In this study we used porcine samples from a previous unpublished vaccine trial. The immunization doses and necropsy results are shown in table 1. Briefly, 8 two-month old piglets were immunized twice with GST/Tsol18 200 µg in 1 mg of Quil A. A Control group with seven animals immunized with the GST leader sequence only in 1mg of Quil A was included in the study. Vaccination was performed intramuscularly on day 0 and day 15. Animals were kept in an animal facility at San Marcos University in Lima. Fifteen days after second vaccination (day 30) animals were orally challenged with one proglottid of *Taenia solium* obtained from treated patients. Pigs were slaughtered three months after challenge and the carcasses were dissected in order to determine the parasite burden. Cysts founded in each animal were categorized as viable (capable of evagination) or degenerated. Serum samples were collected every 15 days. All samples were tested in triplicate. Sera samples were run in triplicate at 1:80 sera dilution. Samples with antibody activities higher than that of the standard curve were further diluted and re-assayed. Specific anti Tsol18 IgG concentration was calculated based on the activity of the standard curve. Correlation between the antibody response and the number of cysts was calculated using Spearman's test.

II.2.6   Assay variability

Inter and intra assay variation were determined as previously described (Hancock and Tsang, 1986). Briefly, to determine the intra assay variation we used the coefficient of variation (CV) of five samples run in separate plates. The inter assay variability

was calculated using a positive reference sample that was run 20 times over a 3-week period.

II.3   Results

II.3.1   Antigen excess

Determination of reagents in excess was done by titration. Fast ELISA Tsol18 antigen titration showed that the saturation of the polysterene beads by antigen occurred at 1 µg/ml (

Figure II.*1*). Therefore, antigen in excess was found to be higher or equal to 1µg/ml.
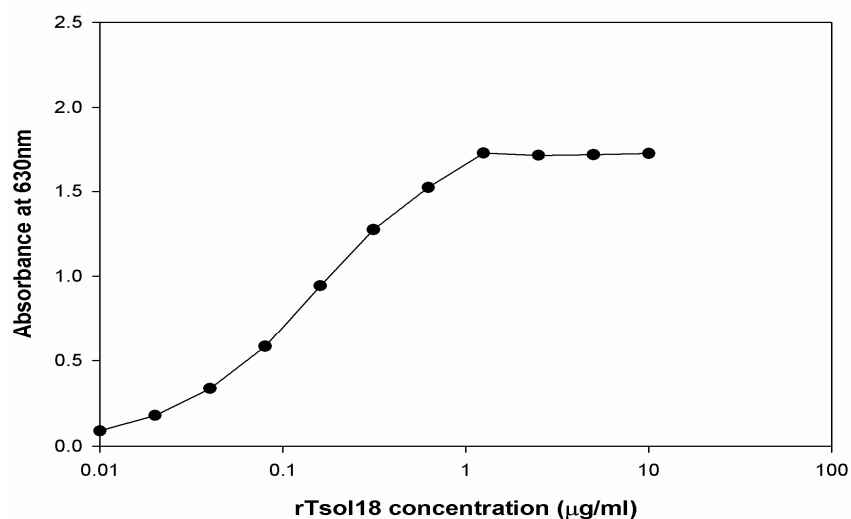
Figure II.1 Recombinant Tsol18 antigen titration.

II.3.2 Standard curve

Normal porcine IgG standard curve was constructed as described above and its activity was measured in the FAST-ELISA format. The test was repeated in three different plates under the same conditions. An 11 point normal IgG standard curve was sensitized to the sticks for 2 hours and then exposed to the conjugate and substrate as described above. We selected a 9 points standard curve (0; 0.05; 0.1; 0.2; 0.8; 1.6; 6.25; 12.5 μg/ml) to be included from now on in each test (data not shown). The standard curve selected had sigmoideal shape that fitted a 4 parameter logistic model with an $r^2 =$ 0.989. This curve was included in all subsequent assays.

II.3.3 Sera titration

Sera titration was performed using antigen and conjugate in excess to establish a optimal assay concentration in order to fit within the dynamic range of the FAST-ELISA. The optimal assay concentration to be used for most sera to be tested by the FAST-ELISA was 1:80 in PBS - 0.3% tween/5% milk. Noise to signal ratio at the chosen dilution was 35 (Figure II.2).
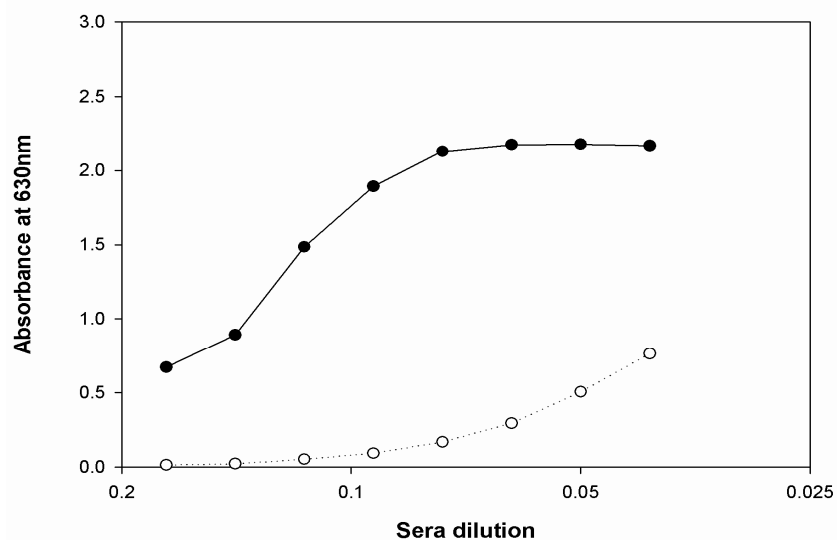
Figure II.2 Porcine sera titration.

Titration of positive and normal porcine pool sera was performed with antigen, conjugate and substrate in excess in the FAST-ELISA format. 2-fold dilutions of each sera starting at 1:5 were exposed to the rTsol18 antigen. The arrow shows the 1:80 sera dilution. Titration of the positive porcine sera is showed in filled circles, the negative in open circles.

II.3.4   Serum

A total of 90 serum samples from 15 piglets from a vaccination trial were as-sayed. Results from the vaccine trial are shown in table 1. Specific serum IgG concen-tration was determined by FAST-ELISA in pigs following immunization with GST-Tsol18 in Quil A. In all pigs vaccinated with GST-Tsol18 (n=8) the antibody response against

rTsol18 showed a similar pattern. Antibodies were detected as early as 15 days after the first immunization and raised to their highest concentration at a mean +/- SE of 5.6 +/- 0.8 µg/ml by day 30 (Figure II.3).

After day 30 the antibody concentration started to decrease. At the end of the study, antibody levels for the vaccinated animals were at a mean +/- SE of 1.6 +/- 0.22 µg/ml. Animals with no viable cyst at necropsy showed an antibody concentration at a mean +/- SE of 5.9 +/- 0.92 µg/ml at the time of challenge on day 30 (Figure II.3). Only one vaccinated animal had viable cysts at the time of necropsy (Table II.1). The amount of antibodies in this animal at the time of challenge was 3.2 µg/ml. The control group (n=7) showed an antibody response of 0.3 +/- 0.1 µg/ml at the time of challenge (Figure II.3).There was no change in the immune response through the experiment in this group. All animals but one in the control group had viable cysts at necropsy, however; in this animal degenerated cysts (651) were found. This animal showed an antibody concentration of 0.9 µg/ml at the time of challenge. The correlation between the antibody responses at the time of challenge and the number of viable and total number of cysts (viable and degenerated) were -0.82 and -0.84 respectively.
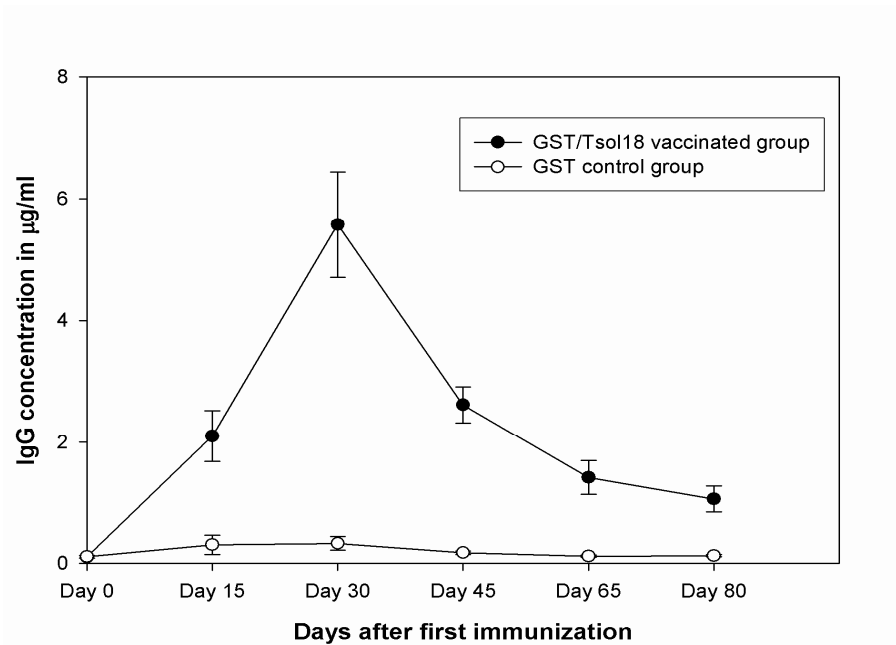
Figure II.3 Specific antibody response in vaccinated and control pigs

Serum samples from 2 months old piglets immunized with GST-Tsol18 were assayed for specific antibody response. Animals were immunized on days 0 and 15 and challenged on day 30. Serum was collected every 15 days. The mean of the antibody concentration +/- SE for each specific day for both vaccinated (n=8) and control (n=7) groups is shown.

Table II.1 Necropsy results from a GST-Tsol18 vaccination trial

Vaccine	Dose	No. of animals	Median of total No. cysts	Minimum – Maximum number of total cysts	Median of No. viable cysts	Minimum – Maximum number of viable cysts
GST (control)	2 doses	7	529	11 - 2185	69	1 - 1755
GST/Tsol18	2 doses	8	1	0 - 82	0	0 - 30

Table shows the median of the total number of cysts per group. The minimum and maximum numbers found per group are also shown.

II.3.5   *Assay variability*

The intra- and- inter assay variability for the FAST-ELISA was determined. The inter- and intra-assay CV were 5.8% and 4.2% respectively. The concentration reading of the positive reference sample in each run must fall within this established CV value to be valid.

II.4   Discussion

The usefulness of the conventional enzyme-linked immunosorbent assay (ELI-SA) as a diagnostic and research tool for either antigen or antibody detection is evident and has been previously revised (Scharpe et al., 1976). However, it has some draw-backs: 1) Long incubation periods: some incubation should be performed for as long as 3 hours. 2) Requires multiple sample dilution titrations which make the assay labor intensive. 3) The substrate incubation exceeds the point where the enzyme concentration is linear with respect of activity and therefore, results can be only considered semi quantitative (Dixon, 1974). 4) Results depend on and endpoint determination in the presence of a stop solution that may not be efficient. 5) Results are reported as the highest dilution generates a color intensity and not as concentration (Tsang et al., 1983a).

The FAST-ELISA is a quantitative assay. Like any other ligand-binding quantitation assay it requires that all the reagents, with the exception of the one being assayed to be used in excess, and that the enzyme activity must be measured when the amount

of substrate present is much greater than that of the enzyme in the reaction (Avrameas, 1976; Avrameas, 1983; Hancock and Tsang, 1986). In the FAST-ELISA a standard curve is used to determine the levels of reactivity of the unknown samples and expressed in concentration units. The use of the standard curve also allows us to use a single dilution of the sera and to compare results from laboratory to laboratory when they are using an equivalent standard curve (Hancock and Tsang, 1986).

The assay we have developed and reported here has several advantages: (1) A single dilution of the sample is assayed. (2) The reactivity of the unknown samples is quantified. (3) Low inter- and intra-assay variation. (4) Using the same standard curve, results are comparable. (5) Only 5 minute incubations. (6) Reasonable cost per test. About US $0.18 per sample run in triplicate (7) Easy to perform and its configuration makes it suitable to be adapted for use in field work.

Taenia solium cysticercosis is a potentially eradicable disease (Aarata A.A, 1992). Control and eradication measures such as identification and treatment of tapeworm carriers, treatment of pigs, education and sanitation have been implemented in field. Despite all these efforts, disease transmission can only be interrupted temporarily and recrudescence occurs as a consequence of tapeworm reintroduction and infection of naïve pigs (Gonzalez et al., 2003). Vaccination of pigs is a measure being tested by us as an adjunct in control programs.

Our optimized FAST-ELISA was able to detect specific anti Tsol18 protective antibodies in vaccinated animals and to follow the immune response up to 80 days after vaccination. The higher concentration of specific IgGs was found on day 30 which was the day of challenge. Additionally, we have found a strong correlation between the antibody concentration and the number of viable and total cysts (-0.82 and -0.84 respectively). The FAST-ELISA as described herein will serve as a useful tool to follow the immune response in vaccinated animals in the field to determine if they are indeed protected.

In conclusion, we have developed a quantitative; simple and rapid assay for detection of specific antibodies against rTsol18. The FAST-ELISA for detection of specific antibodies against rTsol18 can measure the immune response in vaccinated pigs. Moreover, this antibody response strongly correlated not only with the number of both viable and recovered degenerated cysts. The assay we presented here could be helpful for monitoring the immune response of vaccinated animals in field.

III.   A recombinant attenuated *Salmonella* vaccine system for *Taenia solium* cysticercosis infection in pigs

III.1   Introduction

Orally administrated, attenuated strains of *Salmonella enterica* serovar Typhimurium are capable of colonizing gut associated lymphoid tissue and inducing an immune response (Galan, 1996b; Galan, 1996a) . These avirulent *Salmonella* strains have been constructed by introducing mutations in genes required for virulence and modified to express another pathogen antigen in order to be used as an oral delivery vaccine system (Curtiss and Kelly, 1987; Curtiss et al., 1994). Stable and high level expression of heterologous antigens can be achieved using a multicopy plasmid that is stably maintained in the host (Curtiss et al., 1989a; Curtiss et al., 1989b; Curtiss et al., 1990). To enable foreign antigen expression and delivery by the vaccine strain, a balanced-lethal host-vector system has been developed. In this system, the salmonella strain possesses a deletion mutation in the β-aspartate semi-aldehyde dehydrogenase (*asd*) gene coding for diaminopimelic acid (DAP), an essential constituent of the bacterial cell wall and the plasmid vector possesses the wild-type *asd* allele that complements the chromosomal deletion. Loss of the plasmid results in cell death and lysis (Koji Nakayama, 1988).

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Cysticercosis is a parasitic infection produced by the larval stage of *Taenia solium*, which affects pigs and humans following ingestion of the eggs of this cestode (Flisser, 1994; Garcia and Del Brutto, 2000; Sciutto et al., 2000). In humans, the clinical importance of the disease appears when the larvae develop in the central nervous system producing a neurological disorder known as neurocysticercosis (NCC) (Garcia et al., 1999; Garcia et al., 2002a; Garcia et al., 2002b; Garcia et al., 2003a). Although there is an active infection, the disease remains silent until the cysts start to degenerate and induce an immune mediated inflammation which results in clinical manifestation of the disease (Garcia et al., 2002a).

Transmission of the parasite can be interrupted by improving public sanitation, mass treatment of pigs, identification and treatment of tapeworm carriers (Sarti et al., 1992; Sarti et al., 1997; Sarti et al., 2000; Gonzalez et al., 2003; Sarti and Rajshekhar, 2003). However; no control intervention has achieved definitive and sustainable interruption of the *Taenia solium* life cycle. The presence of a susceptible intermediate host permitted reintroduction of the parasite. Vaccination of pigs in combination with the interventions mentioned above may provide the opportunity to definitively interrupting *T. solium* transmission (Lightowlers, 1999). The potential for effective vaccination of pigs has been demonstrated. Recently, Lightowlers and others have shown that the oncosphere antigen known as Tsol18 can confer protection when it is administrated as a recombinant GST fusion protein in 2 doses of 200 µg each (Flisser et al., 2004; Gonzalez et al., 2005). Although Tsol18 has proved to be a good vaccine candidate, the production of a recombinant protein for vaccine use is expensive, and therefore impractical for large-scale use. Therefore, it becomes essential to develop a safe, easy to use and cost

effective vaccine capable to induce long-lasting immune response. In this regard, the use of attenuated *Salmonella* strains to deliver heterologous antigens represents an attractive alternative for vaccine development.

The objective of the present study was to develop a safe, effective, inexpensive and practical delivery system for the protective antigen Tsol18 using an attenuated strain of *Salmonella enterica* serovar Typhimurium as a vector and to test its immunogenicity and efficacy in mice and pigs respectively. For expression of the recombinant protein we used a balanced-lethal host-vector system. In this report we describe a recombinant attenuated *Salmonella vaccine* (RASV) system that is able to express Tsol18, induce a specific humoral immune response against Tsol18 antigen and *Salmonella* antigens in immunized mice and pigs and is capable of reducing the infection burden in vaccinated pigs.

III.2   Materials and methods

III.2.1   Bacterial strains, plasmids, culture media and growth conditions

The bacterial strains and plasmids used for the construction of the recombinant attenuated *Salmonella* vaccine (RASV) against *Taenia solium* cysticercosis are listed in Table III.1 and were kindly provided by Roy Curtiss III, Co-director of The Biodesign Institute at Arizona State University. *Escherichia coli* and *Salmonella enterica* serovar Typhimurium cultures were grown with aeration, at 37 C in Lennox broth or agar (Difco, Detroit MI) supplemented with 0.5% glucose; 0.2% mannose and 0.2% arabinose. When

required; tetracycline was added to a final concentration of 50 µg/ml. Diaminopimelic ac-id (DAP) was added to obtain a final concentration of 50 µg/ml for growth of the *asd* ⁻ strains. Arabinose was depleted from the media to induce expression of the recombinant protein.

Table III.1 Bacterial and plasmid strains used in this study

Bacterial strains or Plasmid	Genotype or characteristic	Source
Plasmid		
pYA3620	3160 bp; *asd⁺*; β-lactamase secre-tion vector.	Curtiss Lab
Strains		
E. coli		
χ 6212/pYA232	F ⁻ λ ⁻ Φ80 Δ(*lacZYA-argF*) *end A1 recA1 hsdR17 gyrA96 relA1 ΔasdA4,* Tetracycline resistant.	Curtiss Lab
S. enterica *Var.*Typhimurium χ9402	*ΔasdA21, Δpmi-2426,Δ(gmd-fcl)-26 ΔP$_{fur81}$,ΔP$_{crp527}$, ΔaraBAD23, ΔaraE25, ΔrelA198, ΔsopB1925*	Curtiss Lab

III.2.2 DNA procedures

DNA manipulations were conducted using standard molecular biology proce-
dures (Sambrook, 1989). PCR amplification was used to obtain DNA fragments for clon-
ing. PCR conditions were as follow: denaturation at 95°C for 30 seconds, primer anneal-
ing at 60°C for 30 seconds, polymerization at 72°C for 30 seconds (30 cycles) and a final
extension at 72°C for 7 minutes. PCR products were visualized in a 1% agarose gel.
Transformation of *E. coli* and *Salmonella* was performed by electroporation (BIO-RAD,
California). Transformants containing the *asd*⁺ plasmid were selected in lennox; 0.2%
mannose; 0.5% glucose; 0.2% arabinose agar plates without DAP. Only clones contain-
ing the recombinant plasmid were able to grow under these conditions.

III.2.3 Recombinant plasmid construction

E. coli 6097/pYA232 was used as intermediate host strains for the cloning. For
antigen delivery to the bacterial periplasmic region plasmid pYA3620 containing the β
lactamase secretion system was used. Mature Tsol18 sequence was amplified (forward
EcoRI: 5' -GGA ATT CGA CCG AAC ATT CGG CGA CGA TAT TTT CGT G- 3'; reverse
PstI: 5' –AAC TGC AGC GAT CTT CGG ACC TTC TTG TGC TTG ATC TGC- 3') and
cloned into pYA3620 to yield plasmid pYA3620/Tsol18. In frame cloning was confirmed
by nucleotide sequencing. After cloning the recombinant plasmid was transformed into
the intermediate host; purified and transformed into *Salmonella*. Success of transforma-
tion was confirmed by PCR and nucleotide sequencing using a specific set of primers

that amplified the recombinant plasmid (Forward: 5'- AAA GAG ATG TGC GGA TCT TCC- 3'; Reverse: 5' TCA GGC TGA AAA TCT TCT CTC ATC- 3').

III.2.4 Expression test

To test the expression of recombinant Tsol18 by *Salmonella enterica* var typhi-murium and *E. coli*, transformed bacteria were grown over night at 37C with shaking at 300 rpm. The overnight culture was diluted 1:50 and grown in the absence of arabinose for 4 hours. Cultures were harvested by centrifugation at 5000 x g, 4C, 5 minutes. The pellet from 50 ml culture was suspended in 50mM Tris/HCl, pH 8.0, 8M urea, 2% SDS, 0.1% bromophenol blue; and boiled for 5 minutes. Proteins were separated by electro-phoresis in a 5% to 22.5% gradient sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE), transferred to a nitrocellulose membrane then washed and exposed to goat anti Tsol18-POD labeled (1:200) (Tsang et al., 1995). Bound antibodies were visualized with DAB (3,3' diaminobenzidine) substrate solution (Tsang et al., 1989).

III.2.5 Plasmid stability

A single colony of the transformant was grown in 3 ml of media supplemented with DAP overnight. A 1:1000 dilution into the same type of media was performed the following day. Inoculated cultures were grown under standard conditions for 14 hours (approximately 10 generations), after which a 1:1000 dilution was performed again. This process was repeated for 5 consecutive days. To determine the proportion of cells re-

taining the *asd*[+] plasmids, dilutions of 10^{-5} and 10^{-6} from each culture after 14 hours of growth were plated onto Lennox agar plates supplemented with 0.5% glucose; 0.2%mannose, 0.2% arabinose and DAP. The following morning, 100 colonies were picked and patched in the same kind of agar plates that were either unsupplemented or supplemented with DAP. Colonies in both plates were counted after 24 hours of incubation and the percentage of colonies retaining the plasmids was determined.

III.2.6 Phenotype characterization

The presence of the Δ*asd*A21 mutation in Salmonella χ9402 was confirmed by the inability of the strain to grow in media without DAP. Lypopolisaccharides (LPS) profiles of *Salmonella* transformants were examined by SDS-PAGE using previously described methods (Hitchcock and Brown, 1983).

III.2.7 RASV preparation

For the vaccine inoculum RASV strains were grown overnight at 37C with aeration. The following day, 100 ml of media were inoculated with 1 ml of the overnight culture (1:100) and grown with aeration (300 rpm) at 37C to an OD_{600} of 0.8 to 0.9. Cells were centrifuged at room temperature (7000 x g for 15 minutes) and the pellet was resuspended in 1 ml of buffered saline with gelatin (BSG).

III.2.8 Mice experiment

Twenty female pathogen free mice were immunized with either one or two doses of *Salmonella* χ9402 harboring pYA3620-Tsol18. Mice were orally inoculated with 50 ul of BSG containing 10^9 CFU. Control groups of four mice each were immunized with *Salmonella* χ9402 containing the empty plasmid. Blood samples were collected every 2 weeks for 10 weeks.

III.2.9 Porcine challenge

To test vaccine immunogenicity and efficacy in piglets; sixteen 2-months old, cysticercosis free piglets were randomly distributed in 2 groups. Piglets were orally immunized with 500 µl of BSG containing 10^{12} CFU. The control group received the salmonella vaccine vector containing an empty plasmid. Four weeks after immunization, piglets were orally infected with one proglottid of *T. solium*. Animals were observed for 2 hours after infection and kept isolated in an experimental animal facility for four months. Serum samples were collected weekly for 12 weeks, thereafter every 2 weeks. Piglets were observed twice a day and a clinical exam was performed on a daily bases.

Ninety days after challenge, all pigs were sacrificed using an over dose of anesthesia. Animals were carefully examined following the standard necropsy procedure. Entire musculature, diaphragm, heart, masseters, tongue and brain were dissected in small slices and examined for the presence of cysts. The total number of cysts in each animal was counted and recorded. Cysts were classified as viable or degenerated based on its

macroscopic appearance. A sample of viable cysts from each animal was tested for *in vitro* evagination in bovine bile (Gonzalez et al., 1997).

III.2.10 Recombinant Tsol18 and Salmonella antigens preparation

Tsol18 mature sequence was cloned into a pET100 vector; expressed in *E. coli* (BL21 Star) (Invitrogen, San Diego, CA) and purified by nickel affinity column chromatography (His-Trap GE Healthcare). The 6X his leader sequence was cleaved with enterokinase (1U/20 μg/30ul) (Invitrogen, San Diego, CA) and removed by affinity purification using a soybean trypsin inhibitor column (Pierce, Rockford IL). The unbound peak containing naked Tsol18 (13.4 kDa) was re-purified by Ni affinity chromatography.

Salmonella outer membrane proteins (SOMPs) antigen was prepared as follows. An overnight culture of *Salmonella* χ9402 was diluted 1:100 in Lennox broth and growth at 37C with aeration (300 rpm) to an OD_{600} of 1. The bacterial culture was then pelleted by centrifugation (6000xg for 15 minutes at 4C). The pellet was resuspended in 10mM HEPES buffer on ice and sonicated. The lysate was centrifuged at 14000 x g for 30 minutes. The pellet was resuspended in HEPES buffer; 2% sodium lauryl sarcosinate (sarkosyl) (Merck) and incubated at room temperature (RT) for 30 minutes. The resuspended pellet was centrifuged again (15000 x g for 30 minutes at 4°C) to separate the solubilized membranes. The pellet containing the SOMPs was washed twice with HEPES and resuspended in the same buffer. Total amount of protein was quantified by Bradford protein assay. Prepared SMOPs were used as antigen to determine the antibody concentration generated in vaccinated and control animals. Commercially available

S. typhimurium LPS (Sigma) was used to determine the antibody concentration against this antigen.

III.2.11 FAST-ELISA

Anti-Tsol18, anti-SOMPs and anti-LPS specific IgG antibodies were quantified by FAST-ELISA as previously described (Hancock and Tsang, 1986). Sticks were sensitized with 2 µg/ml of the antigen (LPS, SOMPs or Tsol18) diluted in 0.05M Tris/HCl, 0.3M KCl, 2.0 mM ethylenediaminetetracetic acid (EDTA) pH 8.0. Mice sera were diluted 1: 33 in PBS; 0.3% tween 20; 5% nonfat dry milk. Affinity purified rabbit anti mouse IgG POD labeled antibody was used as conjugate (Tsang et al., 1995). A standard curve of purified mice IgGs was used for quantification. Specific IgG concentration was calculated based on the activity of the standard curve. Serum samples with absorbance values activities higher than those of the standard curve were further diluted and re-assayed.

III.3 Results

III.3.1 Vaccine construction and Tsol18 expression

We successfully cloned mature Tsol18 sequence into pYA3620 plasmid and transformed into *Salmonella* χ9402. Colony PCR results confirmed successful transformation (data not shown). DNA sequence analysis showed there was no mutation in the clone. The RASV construction expressing recombinant Tsol18 as detected by western blot is shown in Figure III.1 (Tsang et al., 1989)
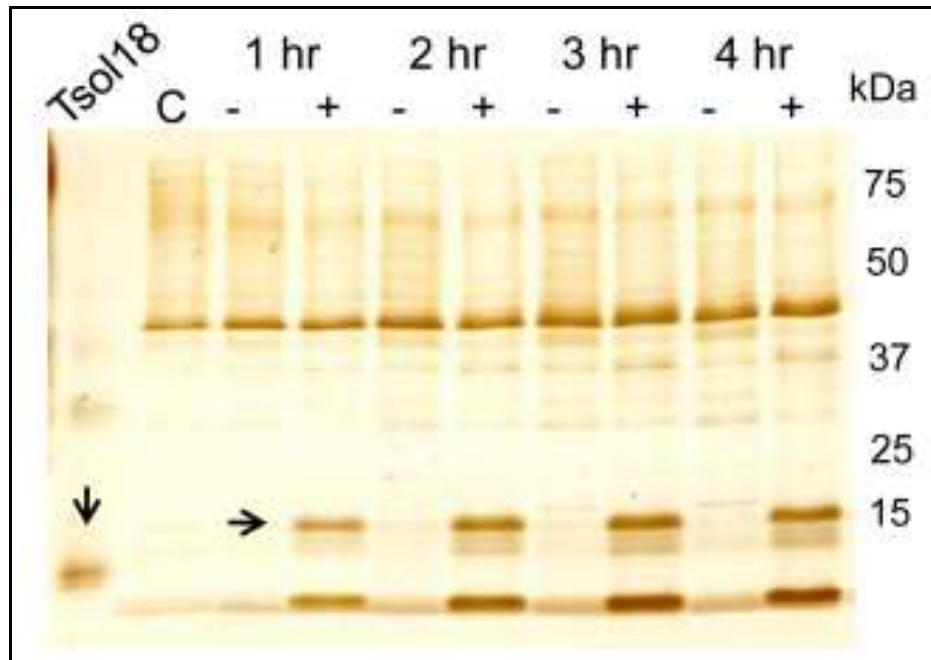
Figure III.1 Recombinant Tsol18 expression by RASV.

Whole cell lysate Salmonella Typhimurium X9402 synthesizing bla - SS- Tsol18 – blaCT fusion protein collected at different time points after inducing with IPTG. (C) Vector control harboring the empty plasmid served as control. Tsol18 = purified recombinant Tsol18 used as positive control; +, induced; -, uninduced. Arrows indicate pure recombinant Tsol18 and the fusion protein in each induced lane.

III.3.2 Plasmid stability

Expression of rTsol18 antigen by our RASV construct was accomplished by introducing a recombinant plasmid encoding the Tsol18 gene. Therefore, plasmid stability becomes a crucial factor for antigen delivery and for the success of the vaccine system. The balanced-lethal system is designed to avoid the use of antibiotic resistance as selective marker. It uses the *asd* gene of *Salmonella enterica* serovar Typhimurium was for plasmid maintenance (Koji Nakayama, 1988). Plasmid pYA3620 complemented the *asd* mutation of *Salmonella* host strain χ9402 and was stably maintained for 50 generations (data not shown). There was no difference between the LPS profiles of χ9402 (with or without the asd⁺ plasmid) and the wild type strain χ3761 (data not shown).

III.3.3 Humoral immune response in mice

The specific humoral immune response (IgG) induced by the RASV against Tsol18, SOMPs and *Salmonella typhimurium* LPS were quantified by FAST-ELISA in both groups of mice. Specific antibodies against Tsol18 were detected as early as day 14 and showed similar patterns in both groups of immunized mice. Mice immunized with a single dose of the vaccine construction (n=7) raised their highest level of antibodies at a mean +/- standard error (SE) of 0.78 +/- 0.16 µg/ml on day 42 (Figure III.2). Mice that received a booster dose on day 7 (n=8), showed a peak of antibodies against Tsol18 on day 28 at a mean +/- SE of 1.26 +/- 0.26µg/ml (Figure III.3). At the end of this experi-

ment on day 70, the antibody levels were 0.29 +/- 0.07µg/ml and 0.35 +/- 0.4µg/ml in animals that received one or two doses, respectively (Figure III.2 and Figure III.3). Both groups of vaccinated mice, and the controls showed similar patterns in the specific immune response generated against *Salmonella* self antigens LPS and SOMPs (Figure III.2 and Figure III.3)
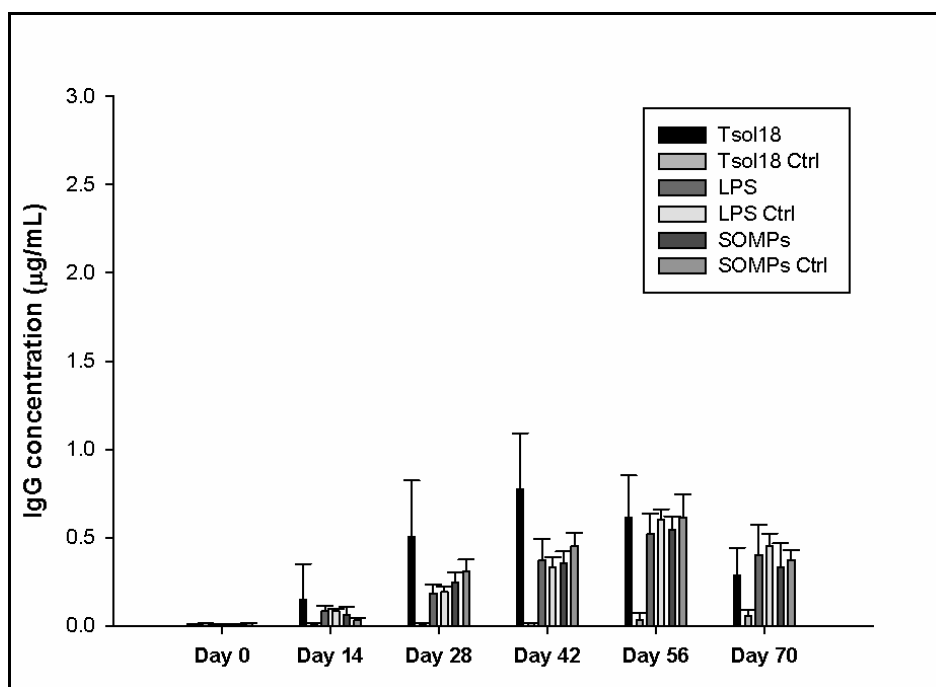
Figure III.2 Specific immune response in vaccinated mice with a single dose of RASV.

Mice were immunized with 10^9 CFU of χ9402/pYA3620-Tsol18 on day 0. IgG antibodies to LPS, SOMPs and rTsol18 were measured by FAST-ELISA in both; vaccinated and control groups. Data is presented as the mean +/- 2 SE.
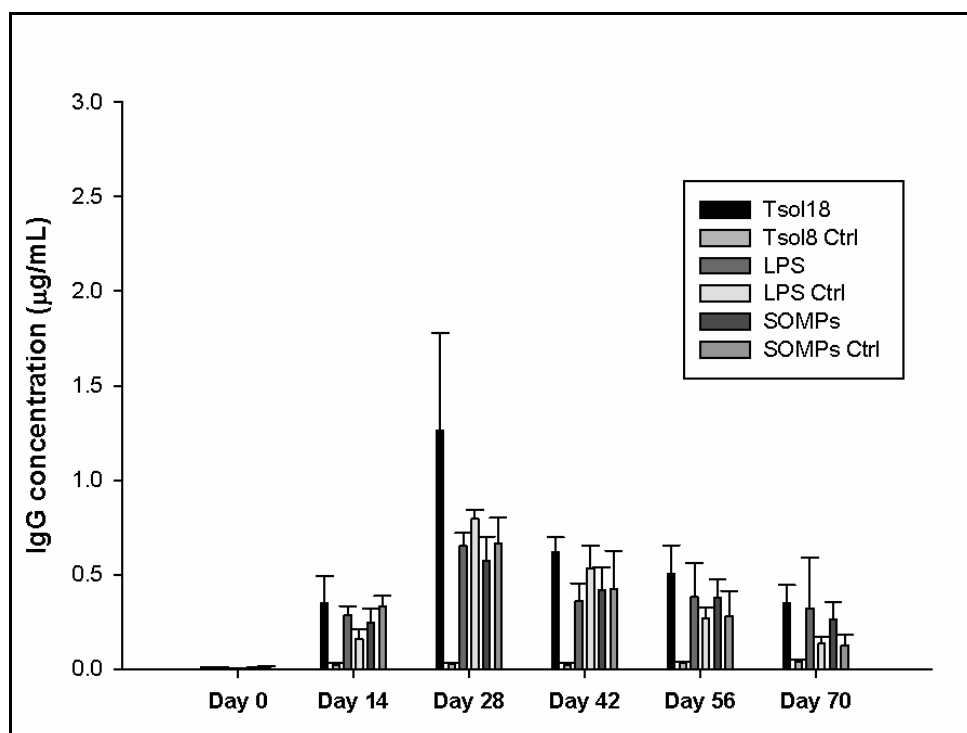
Figure III.3 Specific immune response in mice vaccinated with two doses of RASV.

Mice were immunized twice with 10^9 CFU of χ9402/pYA3620-Tsol18 on days 0 and 7. IgG antibodies to LPS, SOMPs and rTsol18 were measured by FAST-ELISA in vaccinated and control groups. Data is presented as the mean +/- 2 SE.

III.3.4 Humoral immune response in pigs

Specific IgG immune response against Tsol18 antigen was detected in pigs following the immunization with *Salmonella enteric* var Typhimurium expressing Tsol18. Piglets raised their highest level of antibodies against Tsol18 on day 42 at a mean (+/-

SE) of 0.89 +/- 0.05 µg/ml. On this day, the antibody levels against SOMPs and LPS were 0.53 +/- 0.05 and 0.41 +/- 0.02 µg/ml, respectively. The highest means of antibody concentration against SOMPs an LPS in vaccinated animals were found on day 56 (0.69 +/- 0.07 µg/ml and 0.56 +/- 0.05, respectively). The control group also raised their highest specific IgG response against *Salmonella* LPS (0.59 +/- 0.05 µg/ml) and SOMPs (0.67 +/- 0.07 µg/ml) on day 56 (Figure III.4). Specific immune response generated against Salmonella antigens in animals vaccinated with RASV containing the empty plasmid vector was lower than the one generated against Tsol18 in the animals immunized with the construct encoding Tsol18.

Figure III.4 Serum IgG concentration to *Salmonella enterica* serovar Typhimurium LPS, SOMPs and to rTsol18 in immunized pigs

Pigs were orally immunized with 10^{12} CFU of χ9402/pYA3620-Tsol18 on day 0. The control group was immunized with the *Salmonella* strain harboring the empty plasmid. Animals were challenge with one *T. solium* proglottid on day 30. IgG antibodies to LPS, SOMPs and rTsol18 were measured by FAST-ELISA in vaccinated and control groups. Data is presented as the mean +/- 2 SE.
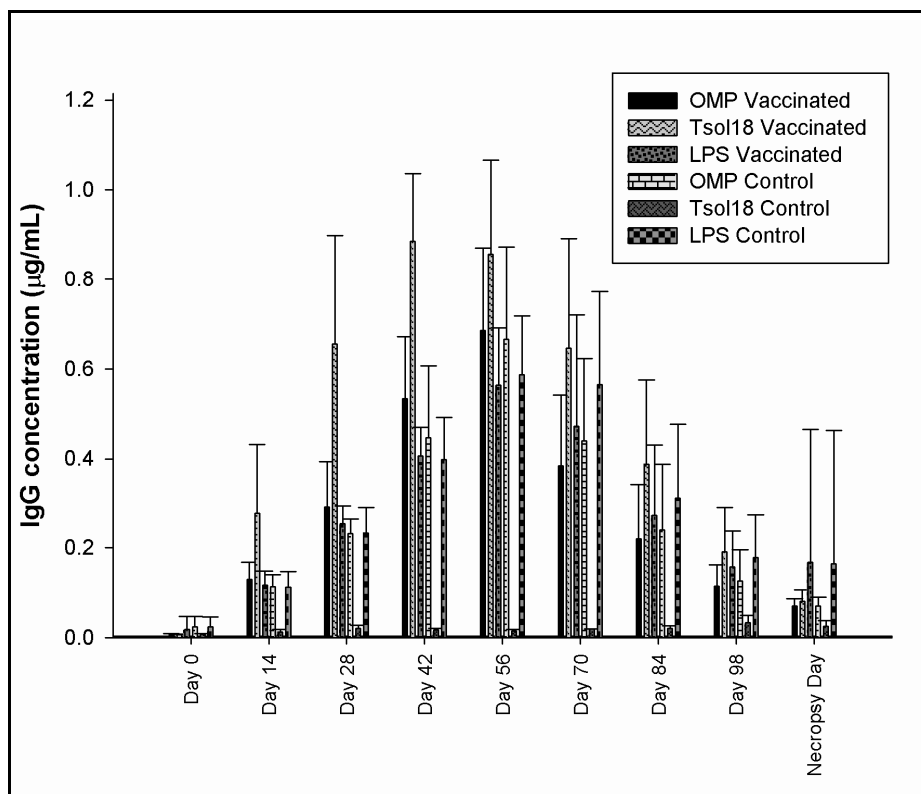
III.3.5 Pig challenge

The vaccine trial was carried out in Peru. All piglets were obtained from a cysticercosis free farm located in Lima. All animals were sacrifice 90 days after challenge and the carcasses were inspected for the presence of cysticerci. The median number of cysticerci found in vaccinated and challenged pigs is shown in Table III.2. The mean numbers of viable and total number of cysts in vaccinated pigs were lower than the numbers in the control group and were statistically significant ($P< 0.05$; Mann – Whitney test). Vaccination with RASV specifying for Tsol18 reduced the number of viable cysts with one pig having no viable cysticercus detected. The total number of cysts was also significantly reduced. Evagination percentage was also lower in the vaccinated group than in the control. Percentages of evagination ranged from 0 to 60% whereas in the control group ranged from 82 to 100%. Clinical observation and clinical exams performed in the experimental animals included body temperature; respiratory frequency; feces consistency, appetite; weight and attitude (alert or depress).   Orally immunized pigs did not showed any health or development problems during the time of duration of the experiment.

Table III.2 Results from the RASV challenge trial in pigs

Vaccine	No. of ani-mals	Median of No. viable cysts	Median of No. total cysts	Evagination (%)
Salmonella				
χ9402-pYA3620 (vector control)	8	542 (307; 2367; 353;731; 89;12; 3616; 3662)	720.5 (663; 2520; 459; 778; 106; 81; 3639; 3399)	93; 82; 95; 96; 87; 100; 100; 100
Salmonella χ9402-pYA3620/Tsol18	8	27.5* (81; 28; 4; 92; 74; 27; 16; 0)	90.5* (157; 41; 340; 104; 77; 73; 43; 120)	66; 0; 0; 60; 67; 7; 0; 0

Table shows the median of the total number of cysts per group (n=8). The numbers of viable and total cysts for each

animal are shown in brackets.

 * Significance (P < 0.05) significance between the number of viable or total cysts in the vaccinated and control group

was calculated using Mann – Whitney test.

III.4 Discussion

Vaccination remains one of the most effective tools for managing infectious diseases in human public health and animal production. The rational development of vaccines requires an understanding of the physiology of the pathogen (Spreng et al., 2006). Ideally, an effective vaccine system should elicit immune responses that result in protection against challenge. In addition, a vaccine system should also be easily administrated, affordable and capable of promoting long lasting protection (Nayak et al., 1998), especially for diseases in economically challenged countries. Furthermore, in the context of cysticercosis, vaccines that are able to stimulate not only systemic but also mucosal immune responses have the advantage of stimulate local immune responses at the port of entry. In this regard, the use of attenuated bacteria that are unable to cause clinical disease but trigger a self-limiting infection leading to the stimulation of protective immunity represents an attractive alternative (Spreng et al., 2006).

Immunity to the larval infection of *Taenia* during the early stages of infection is primarily antibody mediated (Blundell et al., 1968; Gemmell et al., 1968; Blundell et al., 1969). Reports have shown that circulating antibodies generated against metabolic products of metacestodes during early stages of infection are protective. In the particular case of *T. solium* one of such immunogenic antigens is Tsol18, a metabolic product produced during the oncosphere stage of the parasite that have been proved to be protective when used as recombinant antigen (Lightowlers, 2003; Flisser et al., 2004; Gonzalez et al., 2005; Gauci et al., 2006; Kyngdon et al., 2006b). The use of RASV strains to deliver heterologous antigens has advantages over the use of isolated antigen. One ad-

vantage is that the production of the recombinant antigen by the bacteria alleviates the need for an adjuvant. Another advantage is that there is no need for in vitro production or purification of the immunogenic antigen. Finally, the ability of *Salmonella* to colonize the gut lymphoid tissues permits the stimulation of not only mucosal but humoral immune responses (Spreng et al., 2006).

Attenuated *Salmonella* vaccine strains are able to elicit immune responses against antigens that are only expressed in vivo (McGhee et al., 1992). The present study demonstrated that the *T. solium* protective antigen Tsol18 can be expressed in an immunogenic in a *Salmonella* based live vaccine strain. We have cloned mature Tsol18 gene that codes for a 113 aminoacids protein into a recombinant plasmid that harbored the β-lactamase secretion system and transformed into a Δ*crp*, Δ*cya Salmonella enterica* serovar Typhimurium χ9402 strain.

The immune responses to both the heterologous and *Salmonella* self antigens depends on the ability of the RASV to replicate and persist in the host cells. Another important factor in generating specific immune responses is the stability of the recombinant plasmid (Galen and Levine, 2001). Our vaccine construction well tolerated by mice and piglets; and was able to induce a specific immune response against Tsol18 in both groups of vaccinated animals. Serum from immunized animals with the RASV strain reacted with purified Tsol18, suggesting successful delivery of the antigen and induction of humoral immune response. The ability of strain χ9402 (pYA3620-Tsol18) to produce Tsol18 at immunogenic concentrations was probably an important element of its ability to elicit high level of specific IgG response to Tsol18. Higher specific anti Tsol18 IgG was

found in both, mice and piglets on day 42 after immunization. Specific anti Tsol18 anti-body concentration decreased rapidly and by day 98 after immunization the antibody concentration was 0.19 +/- 0.04 µg/ml (Figure III.2; Figure III.3 and Figure III.4). Both experimental groups of animals showed higher antibody concentration against Tsol18 than against *Salmonella* OMPs and LPS (Figure III.2; Figure III.3 and Figure III.4). The observation that the anti-LPS and anti-SOMPs IgG concentrations induced by the vaccine construction (χ9402/pYA3620-Tsol18) and those generated by the same strain harboring empty plasmid in the control groups were comparable indicated that the expression of Tsol18 did not affect the immunogenic potential of the bacteria.

We also tested the efficacy of the RASV to induce protective immune response through a vaccination and challenge trial. Although our trail challenge did not achieve a 100% of protection in vaccinated animals as it was previously reported with another vaccine system (Flisser et al., 2004; Gonzalez et al., 2005), we did significantly reduce ($P < 0.05$) the number of healthy cysts in vaccinated animals with a single dose of our RASV. Reductions of the amount of viable cysts and in its ability to evaginate are of epidemiological importance as they are the source for human infection (Table III.2). There was only one animal with no viable cysts, however; the healthy cyst found in the other pigs during carcasses inspection had their ability to evaginate reduced to levels ranging from 0 to 60%.

In conclusion, heterologous expression of Tsol18 a protective antigen against *T. solium* cysticercosis was demonstrated in *Salmonella enterica* serovar Typhimurium χ9402. The balanced-lethal vector used to clone and express the immunogenic protein

was stably maintained in the system. We have also demonstrated the ability of the vaccine system to elicit specific IgG response in immunized mice and piglets. Moreover, we have demonstrated the ability of the RASV system of delivering protection to pigs using a single dose. Our data together suggest that our RASV is highly effective delivering *Taenia solium* Tsol18 antigen and to induce specific immune response to reduce infection burdens in infected animals when it is orally administrated in a single dose.

IV.   CONCLUSIONS

Many immunological aspects of the relationship between *Taenia sp.* Metacestodes and their hosts have been delineated as a result of investigations performed during the early 1930's. From these studies the following principals have been demonstrated to be applicable to all *Taenia* metacestodes, including *Cysticercus cellulosae*:

1. Infection lead to immunity to re-infection (Miller and Gardiner, 1932; Kerr, 1935).

2. Naïve hosts can be protected by immunization of with metabolic products obtained from non living parasites (Miller and Gardiner, 1932; Campbell, 1936; Campbell, 1938b).

3. Naïve hosts cab by protected by passive transfer of serum from an infective or actively immunized animal (Campbell, 1938b; Campbell, 1938a).

4. During early infections, protection properties of passive transferred serum is associated with IgG antibodies (Leid and Williams, 1974b; Leid and Williams, 1974a; Musoke et al., 1975).

5. Antiparasitic effect on immunized animals have the ability to kill activated oncospheres cultivated in vitro (Kyngdon et al., 2006b).

These principals obtained from early investigations in the immunobiology of Taenia infections set the scene for vaccine development. Previous studies have shown that a metabolic protein known as Tsol18 produced by the larval stage of *Taenia solium* is capable of inducing strong circulating antibodies. Moreover this strong immune response

have demonstrate to be protected for vaccinated animals (Flisser et al., 2004; Gonzalez et al., 2005). Although this metabolic product have demonstrated to be protective when used as recombinant protein, it use have some disadvantages:

- Production and purification of recombinant proteins are expensive and require expensive reagents and equipment.
- Tsol18 used as a recombinant antigen needs to be injected twice into the host in order to protect vaccinated animal. Vaccination through injection requires animal handling that is not always available under field conditions.

Genetic manipulation of *Salmonella* species has resulted in the loss of their pathogenicity without interfering with their ability to stimulate the immune system. In this regard, attenuated *Salmonella* has the ability to generate a strong mucosal immune response in addition to the humoral and cellular immune responses not only against self antigens, but also against recombinant ones (Dougan et al., 1989). Attenuated and immunogenic strains of *Salmonella* can be genetically engineered to stably express foreign antigens. Recombinant strains used as vaccine delivery systems, usually have a plasmid vector that harbors the sequence for the foreign antigen (Chatfield et al., 1993; Chatfield et al., 1994). Several foreign antigens from pathogens closely related to *Salmonella* and also from viruses, or parasites have been expressed in recombinant avirulent *Salmonella* and tested for safety and efficacy against challenge in animal models. Good immune responses were observed when the foreign antigen is retained in the cytoplasm of the recombinant *Salmonella* as well as when foreign antigens are localized to the periplas-

mic space, or to the outer membrane (Khan et al., 1994; Chabalgoity et al., 1996; Chabalgoity et al., 2000; Kang et al., 2002)**.**

The RASV system expressing Tsol18 protective antigen and capable of inducing protective antibodies in vaccinated piglets developed in this research bring some advantages over the use of an isolated recombinant antigen as the one described above:

- The use of a bacterial system to deliver the recombinant antigen alleviates the need for an adjuvant.
- Purification of the antigen is no longer required; therefore, production of the vaccine becomes cheaper.
- The ability of the RASV system to colonize the host gut associated lymphoid tissue without producing any disease, permits not only strong stimulation of specific humoral immune response but also local immune response at the point of parasite entry.

The vaccine construction presented in this research was capable of express Tsol18 antigen and was stably maintain in *Salmonella* up to 50 generations. This construction was also capable to of elicit specific humoral immune response not only against the recombinant Tsol18 but also against Salmonella self antigens such as SOMPs and LPS. Moreover quantification of the specific antibody response by FAST-ELISA showed that the amount of antibodies generated against Tsol18 where higher than those generated against SOMPs or LPS. The efficacy trial performed as a fundamental part of this research showed a significant reduction in the numbers of viable cysts present in vacci-

nated pigs when compared with the control group. Vaccination not only reduced the number of viable cysts but also their ability to evaginate. This reduction in the number of viable cysts and in the evagination rates becomes of epidemiological importance as healthy cysts are the source of human infection and the way for disease perpetuation.

This work presents 2 important tools that can improve *Taenia solium* control programs in field: An effective, feasible, safe, inexpensive and easy to use vaccine system for field use and an quantitative, simple, inexpensive and rapid immological test able to measure the amount of specific antibodies against Tsol18 generated in vaccinated animals that can be use as a tool to follow the immune response in vaccinated animals in field and determine if they are protected.

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