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**IMMUNOREACTIVE PROTEINS IN  
*TAENIA SOLIUM*/*CYSTICERCUS*  
*CELLULOSAE***

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# IMMUNOREACTIVE PROTEINS IN *TAENIA SOLIUM*/ *CYSTICERCUS CELLULOSAE* THESIS FOR LICENTIATE DEGREE

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I dedicate this thesis to my family, especially to my husband Feliciano Lecuane, my son Harriston Lecuane and Ivanylson Lecuane.



## ABSTRACT

Cysticercosis caused by *Taenia solium* is a common zoonotic parasitic disease in Latin America, Asia, Africa, and parts of Oceania, and it is responsible for many cases of epileptic seizures. Imaging techniques used to diagnose neurocysticercosis (NCC) are expensive and therefore rarely available in endemic countries. Several reliable methods have been developed for diagnosis of cysticercosis, and these include the use of recombinant antigens. Commercial antigens for diagnosing human NCC are obtained from either a soluble parasite extract or a parasite-derived glycoprotein fraction.

In this study, we identified and evaluated the antigens Tsol-p27 (previously detected in Nicaragua) and cC1 as potential recombinant diagnostic reagents, and we also investigated the localization and partial function of Tsol-p27. Immunoblotting using recombinant Tsol-p27 revealed that Tsol-p27 was recognized by antibodies in all 10 analysed serum samples from NCC-positive individuals, whereas recombinant cC1 was recognized in only five of the 10 Tsol-p27-positive sera. None of the negative control sera showed a positive reaction to either of the recombinant antigens.

We also used immunoelectrotransfer blot (EITB) to analyse serum samples from 165 people in Mozambique. This assessment confirmed that 18 of the subjects were NCC positive and the remaining 147 were NCC negative. Furthermore, Western blot analysis of Tsol-p27 showed 85.71% sensitivity and 96.69% specificity.

Despite the limited number of serum samples evaluated in the present studies, the results suggest that Tsol-p27 antigen provides good sensitivity and specificity and can thus be considered a suitable candidate for diagnosis of human NCC in both Central America and sub-Saharan Africa.

## LIST OF SCIENTIFIC PAPERS

- I. Further characterization of Tsol-p27 as a diagnostic antigen in sub-Saharan Africa. *Exp. Parasitol.* 2013 Nov; 153(3): 573–579.
- II. Performance of Tsol-P27 antigen for serological diagnosis of cysticercosis in Mozambique. *J Helminthol.* 2015 Aug 21:1-4.

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## ABBREVIATIONS

2-DE	two-dimensional electrophoresis
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulphonate
CNS	central nervous system
CT	computed tomography
DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
<i>E. granulosus</i>	<i>Echinococcus granulosus</i>
<i>E. multilocularis</i>	<i>Echinococcus multilocularis</i>
EITB	enzyme-linked immunoelectrotransfer blot
ELISA	enzyme-linked immunosorbent assay
ESA	Eastern and Southern Africa
GST	glutathione S-transferase
IEF	isoelectric focusing
Ig	Immunoglobulin
IPG	immobilized pH gradient
IPTG	isopropyl B-thiogalactopyranoside
kDa	kilodalton
LC–MSMS	liquid chromatography–mass spectrometry
NCC	Neurocysticercosis
NCBI	National Center for Biotechnology Information (USA)
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

# 1 INTRODUCTION

## History

Cysticercosis and taeniasis were known in antiquity but described in the literature only a few centuries ago. Aristophanes and Aristotle referred to porcine cysticercosis in 380–375 BC when they described the occurrence of cysticerci in the human tongue detected by palpation [1]. An Egyptian papyrus document from 1500 BC made allusions to illnesses known at the time, one of which was no doubt caused by *Taenia solium*. Also, ancient Hebrew Scriptures prohibited the consumption of pork, because most early Jewish people had a boar as a totem and probably had no idea of the role that parasites play in human health [1, 2].

The first case of human cysticercosis to be clearly identified was reported in 1558, and cysticerci was recognized as parasite in 1697. A link between taeniasis and cysticercosis in the same patient was observed in 1782, and the life cycle of *T. solium* was described three years later [1, 2].

## General background

Taeniasis and cysticercosis constitute an important public health problem because they occur primarily in low-income countries, where the persistence of the infection is promoted by various cultural and socio-economic factors, as well as poor sanitary conditions [3]. *Taenia* infection is endemic in many countries in Central and South America, Asia, Africa, and parts of Oceania, affecting thousands of individuals and representing the major cause of epilepsy worldwide [3, 4].

Humans and pigs can develop cysticercosis by ingesting eggs of the zoonotic cestode *T. solium*. Cysticercosis is one of the few infectious diseases that can be eradicated, and it is the target of control programmes in many countries [5]. The fact that *T. solium* cysticercosis is often overlooked as a possible cause of epilepsy, along with the scarcity of reliable diagnostic techniques, explains why human cysticercosis remains an underestimated public health problem [6]. One of the main difficulties associated with controlling *T. solium* cysticercosis is that people in endemic areas lack knowledge about this infection. In short, the majority of individuals in those regions are not aware of the relationship between the intestinal

tapeworm in pigs (known as “solitaria”) and cysticercosis in pigs and humans, and hence they do not understand the danger in of eating *Cysticercus*-infected pork, and some even prefer such meat for its particular flavour [7].

Neurocysticercosis (NCC) is caused by the presence of *T. solium* larvae in the central nervous system (CNS). NCC is the most widespread parasitic disease of the human brain, and it is responsible for most cases of adult-onset epilepsy in the world [8]. It has been estimated that annual work productivity is reduced by up to 30% in people with NCC-induced epilepsy [9].

## 2 ETIOLOGY

### Morphology

The pork tapeworm belongs to the kingdom Animalia, the phylum Platyhelminthes, the class Eucestoda, the order Cyclophyllidea, and the family Taenidae, and it has been given the genus and species names of *Taenia solium* [10]

**The adult tapeworm:** In this stage, *T. solium* is a flat, segmented, hermaphroditic parasite that is 2 to 10 meters long and is found in the human small intestine. The worm is composed of a head or scolex that is approximately 1 mm in diameter and bears four muscular suckers that enable attachment and to some extent also locomotion [11, 12]. Anterior to the suckers is an armed rostellum armed with 22 to 36 hooks ordered in two rows. A thin neck about 5 to 10 mm in diameter is responsible for most of the biokinetics of the tapeworm, as well as formation of the strobila that make up the rest of the body [12]. The strobila consists of between 800 and 4,000 proglottids or segments, which can be grouped as immature, mature, and gravid. Immature proglottids are wider than they are long; mature proglottids are square in shape and contain mature primary sexual organs; gravid proglottids are rectangular in shape (longer than wide) and most of their sexual organs are atrophied, and they are filled almost entirely with branched uteri packed with spherical eggs called oncospheres [11].

**Oncospheres:** Oncospheres are 29 to 77  $\mu\text{m}$  in diameter. An oncosphere is composed of a chorionic membrane, a thick and grooved embryophore surrounded by embryophoric blocks (consisting of keratin-like protein), an embryophoric membrane, and two oncospherical membranes that envelope the oncosphere (also called a hexacanth embryo), which bears three pairs of hooks [11].

**Cysticerci:** Cysticerci located in the brain and muscle of both humans and pigs exhibit the same morphological characteristics. A cysticercus consists of an ovoid vesicle that is approximately 5–15 mm in diameter, has a transparent outer membrane, and is filled with a colourless fluid and contains an invaginated scolex (with four suckers and an armed rostellum with two rows of hooks). The wall of the cysticerci is a membranous structure composed of a cuticle or external layer, a cellular or middle layer, and a reticular or internal layer [13].

## Life cycle

The life cycle of *T. solium* involves two hosts (Fig. 1): humans are the only definitive hosts for the adult tapeworm, whereas both pigs and humans can serve as intermediate hosts for the larval form. In the normal cycle of transmission, the adult *T. solium* inhabits the small intestine of humans, where it is attached to the intestinal wall by its strong suckers and hooks. Gravid proglottids are detached from the distal end of the worm and are passed in the host's faeces to liberate thousands of fertile eggs in the surrounding environment. In low-income areas with poor sanitation, the food that is eaten by pigs is often contaminated with human faeces containing *T. solium* eggs [14].

After *T. solium* eggs have reached the intestinal tract of a pig, they lose their protective coats and liberate oncospheres that cross the intestinal wall and enter the pig's bloodstream, and are subsequently carried to the tissues and evolve into cysticerci. If improperly cooked infected pork is consumed by a human, cysticerci are released in the small intestine, where, under the action of the host's digestive enzymes, the scolices evaginate and attach to the intestinal wall. Thereafter, the proglottids begin to multiply and become mature tapeworms within approximately four months [15].

Humans can also be intermediate hosts for *T. solium*, and such infection is called human cysticercosis/neurocysticercosis [4]. Cysticercosis can occur through ingestion of food contaminated with *T. solium* eggs or by the faecal-oral route in a human harbouring an adult *T. solium* in the intestine. Indeed, recent epidemiological studies have shown clustering of patients with cysticercosis around individuals with taeniasis, which has changed previous concepts that pinpointed the environment as the main source of human contamination with *T. solium* eggs. Thus human cysticercosis should be considered as an infection transmitted mainly from person to person, and the role of infected pork is to perpetuate the infection [5]

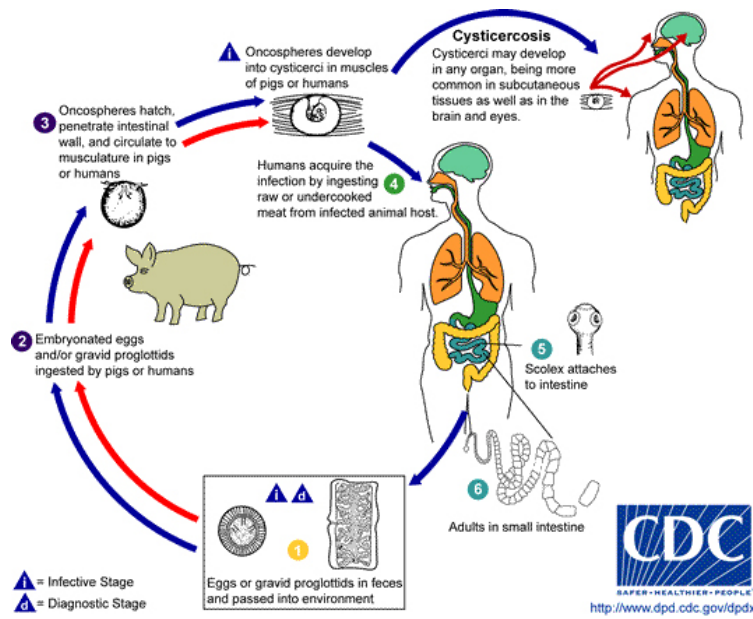


Figure 1. The life cycle of *Taenia solium*. (From CDC: <http://www.dpd.cdc.gov>)

### 3 CLINICAL ASPECTS

#### **Taeniasis**

The most noticeable sign of taeniasis is the presence of proglottids either in the faeces or actively migrating out of the host. A few gravid *T. solium* are released in the host's faeces once a day or two or three times a week. Taeniasis per se causes low morbidity and is usually asymptomatic, although people with the disease have reported prurits in the peri-anal region, abdominal pain, distension, nausea and diarrhoea [16].

#### **Human cysticercosis**

Neurological symptoms are the most important characteristic of human *T. solium* cysticercosis. About 50–70% of patients with cysticerci localized to the CNS are asymptomatic, and epilepsy is the most common clinical manifestation of NCC in symptomatic patients [17]. Additional symptoms described are nausea, migraine, mental disorders, nervous hypertension, endocrinal hypertension, and paralysis [18], [19].

Subcutaneous cysticercosis entails typical larvae-containing nodules that are painless for the host but surrounded by varying degrees of inflammation. Cysts in muscles cause severe pain, especially if the parasite load is heavy, and the larval stage can also become established in the myocardium [20]. In the eyes, retinal and vitreous involvement occur most frequently, followed by sub-retinal, conjunctival, and anterior segment localization, and the first-mentioned can lead to uveitis, iritis, and retinitis and can also affect the muscles of the eye [21].

#### **Porcine cysticercosis**

Cysticercosis-related symptoms are usually not manifested when cysticerci are present in muscles or adjacent tissues. Nevertheless, reports indicate that common symptoms in experimentally infected pigs include anorexia, fever, bradycardia with increased respiratory rate, nausea, and diarrhoea, as well as abortion and death in cases of massive infestation [22], [23].

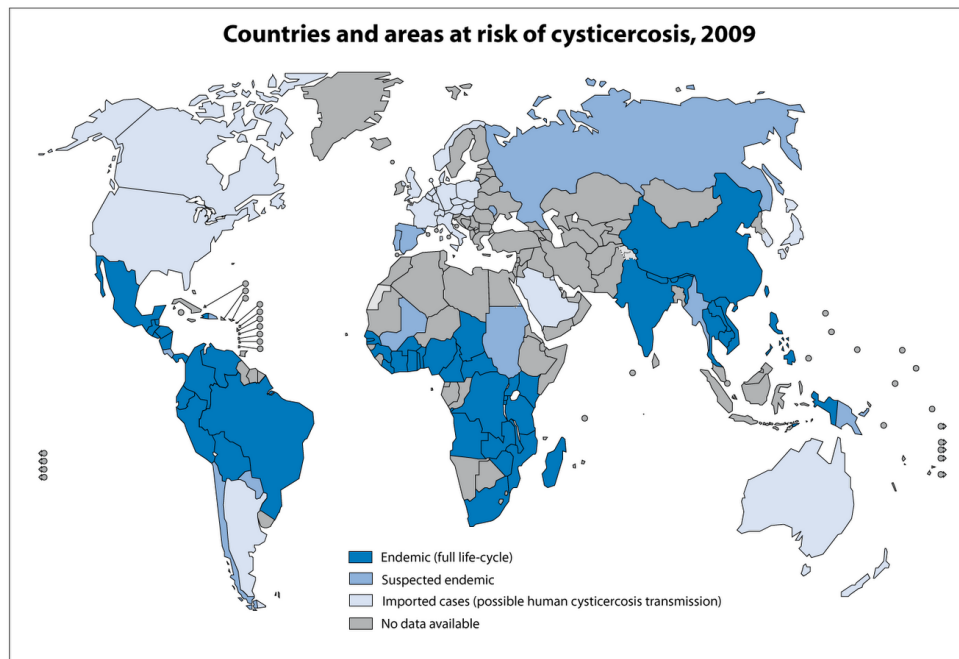
## 4 EPIDEMIOLOGY

Human cysticercosis is associated with resource-limited settings where people eat raw or undercooked pork and traditional pig husbandry is practised. Furthermore, transmission of the disease is facilitated by poor hygiene and inadequate sanitation, as well as the use of untreated or partially treated wastewater in agriculture [24]. Today, the regions with the highest prevalence are Latin America, Southeast Asia, Africa, and Eastern Europe, and therefore tourism and immigration from endemic countries has led to emergence of cysticercosis in some high-income countries [25].

*T. solium* cysticercosis is widely distributed in many parts of Africa where it is customary to raise pigs. Swine production has increased significantly in the Eastern and Southern Africa (ESA) region during the past decade, especially in rural, resource-poor smallholder communities [6]. Concurrent with the rise in smallholder pig keeping and pork consumption, there have been increasing reports of porcine and human cysticercosis in Western and Central Africa and the ESA countries [6], [26].

In Mozambique, swine production is driven predominantly by smallholders, particularly in the central part of the country. Pigs are generally kept under extensive (free-range) conditions in which the animals obtain food primarily by scavenging and normally have access to primitive housing only at night. Inspection and control of pork is limited due to lack of slaughterhouse facilities. Together, these circumstances favour perpetuation and spreading of *T. solium*, which poses a serious public health risk for the population in general [27].

Data concerning human cysticercosis in Mozambique are scarce. In one investigation [28], anti-*Cysticercus cellulosae* antibodies were detected in 20.8% of the children living in the urban environments of Maputo. Another study conducted in 1993 showed that 12.1% of patients and visitors at Maputo Central Hospital were positive for antibodies against *Cysticercus cellulosae* [29].



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. © WHO 2010. All rights reserved

Data Source: World Health Organization  
Map Production: Control of Neglected  
Tropical Diseases (NTD)  
World Health Organization



Figure 2. The epidemiology of cysticercosis. (From WHO:

[http://gamapserver.who.int/mapLibrary/Files/Maps/Global\\_cysticercosis\\_2009.png](http://gamapserver.who.int/mapLibrary/Files/Maps/Global_cysticercosis_2009.png))

## 5 DIAGNOSIS

### Taeniasis

Several methods have been developed to identify intestinal taeniasis. Macroscopic diagnosis entails searching for proglottids in faeces or recovery of a worm after treatment. Routine diagnosis is based on microscopic detection of *Taenia* spp. oncospheres in direct smears or after concentration performed using various approaches, such as Kato/Katz and formol-ether methods, that have a sensitivity of around 38% to 60% [3]. However, these methods do not allow differentiation between *T. solium* and *T. saginata*, which instead requires identification of adult tapeworms or proglottids [30]. Notably, highly specific polymerase chain reaction (PCR) methods for detection the DNA of different *Taenia* species were developed a few years ago, but those assays have not been properly validated in the field [31, 32].

### Cysticercosis

Ante mortem diagnosis in pigs is often done by visual inspection and tongue palpation. Due to their coprophagous habits, pigs are often massively infested with cysticerci in all parts of the body, including the brain [33]. Both ante and post mortem examinations offer high sensitivity and specificity if the animals have extensive infections, whereas those techniques provide considerably lower sensitivity when applied to pigs with limited infections [34].

Immunodiagnostic methods have been developed that use antibodies to detect *T. solium* cysticercus antigen in serum and cerebrospinal fluid from humans and animals. EITB for cysticercosis is regarded as the gold standard assay for serological identification of this infection and is considered to offer the highest sensitivity and specificity [34-36]. However, this test is based on a complex of native proteins employed in immunoblot assay formats, which have not yet been successfully adapted for use under field conditions. Due to the high cost of EITB, several enzyme-linked immunosorbent assays (ELISAs) for antibody detection have been developed that use crude antigens, purified *T. solium* antigens, and synthetic

peptides [37, 38]. These assays have shown high sensitivity and specificity, but they are only able to identify presence of the vesicular larval stage.

In recent years, the use of magnetic resonance imaging (MRI) and computed tomography (CT) has enabled diagnosis of NCC cases with a benign course that would not have been detected previously. These techniques allow detailed assessment of viable, degenerated, and calcified cysticerci or lesions in the human CNS, but they are expensive and not widely available in endemic countries, where there is also a scarcity of skilled experts to interpret CT and MRI images [39].

## 6 PREVENTION AND CONTROL

In 2002, the World Health Organization (WHO) formulated measures to control NCC based on the following knowledge [40]: the larval stage of the pork tapeworm is the causative agent of cysticercosis; cysticercosis is the most important parasite-induced neurological condition in humans; human cysticercosis is associated with poverty in areas where pigs are raised under free-range conditions; cysticercosis of the CNS is an important cause of chronic epilepsy. Notably, several characteristics of cysticercosis make it a potential target for eradication: humans are the only definitive host of the parasite; humans carriers are the sole source of infection for intermediate hosts; transmission of the infection from pigs to humans can be controlled; there are no important wild reservoirs of the parasite [41, 42]. In addition, there are practical methods for surveillance in pigs (e.g., tongue palpation), and EITB and copro-antigen techniques can be used in humans. Moreover, there are safe and effective drugs for mass chemotherapy of taeniasis and cysticercosis in pigs [43].

Efforts should be made to reduce of *T. solium* by improving human hygiene and sanitation. Examination of the tongues of pigs in slaughterhouses represents an inexpensive strategy to rapidly detect infection foci and thereby determine where interventions are needed. Farmers must be convinced that it would be of financial benefit to raise pigs more hygienically, and evidence is required to support that assertion [43]. In a study conducted in the Angonia District in Mozambique [27], it was found that porcine cysticercosis could be effectively controlled by treating pigs with oxfendazole, but it was also concluded that such an approach needs to be integrated with other control measures, including health education for the target population and treatment of human tapeworm carriers. In Mozambique, a serious constraint is that national programmes to control or eliminate cysticercosis are lacking due to economic obstacles in the form of slaughterhouse facilities, poor sanitary conditions, and scarcity of affordable diagnostic methods.

## 7 AIMS

The general objective of the studies included in this thesis was to develop and improve methods for diagnosis of cysticercosis. More specific aims were as follows:

- To identify immunoreactive proteins from *Taenia solium* cysts (Paper I).
- To validate an immunodiagnostic test for human cysticercosis (Paper II).

## 8 MATERIAL AND METHODS

### Source of antigen

**Paper I:** Intact *T. solium* cysts used for determination of immunogenic proteins were obtained from naturally infected pigs raised in an endemic area of Mozambique. The cysts were washed with phosphate-buffered saline (PBS; pH 7.5) and kept at  $-80^{\circ}\text{C}$  until used. Briefly, cysticerci were mechanically disrupted in 500  $\mu\text{l}$  of PBS and homogenized with a protease inhibitor cocktail (Invitrogen®). The sample was subsequently centrifuged at  $13,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ , and the supernatant was stored at  $-20^{\circ}\text{C}$ .

### Sources of human sera

A total of 181 serum samples were collected from patients in Mozambique as described below.

**Paper I:** Sera were obtained from 20 epileptic patients who lived in Tete Province and were found to be positive or negative for NCC by Ag ELISA. The patients were also examined individually by computed tomography (CT) and, based on the results of both tests, their serum samples were divided into a NCC-positive group ( $n = 10$ ) and a NCC-negative control group ( $n = 10$ ) [7].

**Paper II:** One hundred sixty-five serum samples from patients in Tete and Sofala Provinces were used to evaluate the efficacy of an immunoblot method.

### Two-dimensional electrophoresis (2-DE)

**Paper I:** IPG strips with a linear pH range of 4–10 or 4–7 (GE Healthcare) were rehydrated for 12 h according to the manufacturer's protocol. The extracted proteins were mixed with rehydration buffer containing 6 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.4% (w/v) DTT, and 0.002% (w/v) bromophenol blue. The isoelectric focusing (IEF) was performed using a Multiphor system (Pharmacia Biotech) and the following conditions: 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2,000 V for 30 min. In the second dimension, the strips from IEF were loaded for sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE; 12%) according to the protocol provided by the manufacturer (Pharmacia Biotech).

### **Western blot analysis and protein sequencing**

**Paper I:** After 2-DE, the acrylamide gels were directly stained with Coomassie Blue (Bio Rad) or were transferred to nitrocellulose membranes (GE Healthcare). Membranes blotted with proteins separated by 2-DE or proteins blotted with recombinant cC1 or recombinant Tsol-27 antigens were blocked for 2 h with 5% skim milk in PBS (blocking solution) and subsequently rinsed in PBS and 0.05% Tween 20 (washing solution). Thereafter, the membranes were incubated for 3 h with human sera diluted 1:500 in blocking solution and then for 1 h with rabbit anti-human IgG conjugated with peroxidase (Sigma) diluted 1:3,000 in blocking solution. Detection was performed according to the ECL plus manual (GE Healthcare).

The blotted proteins that reacted with positive sera were identified by comparing with Coomassie-stained gel. The protein spots exhibiting the greatest immunoreactivity were selected, excised from the gel, and stored at  $-20^{\circ}\text{C}$ . Later, the excised spots were trypsinized, and the proteins were subjected to amino acid sequencing by liquid chromatography–mass spectrometry (LC–MS).

### **Cloning, expression, and purification of recombinant proteins**

**Papers I and II:** Recombinant Tsol-p27 and cC1 proteins were produced from selected cDNA and amplified using specific primers. For cC1, we used the primer pair cC1F 5'- CTC GGA TCC ATG GCC TAC TGT CGC TCC CTG- 3' (sense)/cC1 R 5'-CGG GAA TTC CGG TGC AGG GCC GAT GAG TTT CA-3' (antisense). The Tsol-p27 protein was expressed as previously described [44]. All PCR experiments were performed using a Thermo Hybrid system and Maxima Hot Start PCR 2xMaster Mix (Fermentas). The PCR conditions were as follows: one cycle at  $96^{\circ}\text{C}$  for 5 min followed by 30 cycles at  $96^{\circ}\text{C}$  for 45 s,  $52^{\circ}\text{C}$  for 45 s,  $72^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 7 min. The cC1-amplified ORF was cloned into pTrcHis2 C (Life Technologies) and expressed as a recombinant protein. The recombinant cC1 was purified using his-selected nickel magnetic beads (Sigma). The resin was equilibrated with a buffer containing 50 mM sodium phosphate (pH 8.0), 0.3 M

sodium chloride, and 10 mM imidazole, and then eluted with a higher concentration of imidazole (250 mM). The histidine-containing proteins were separated from the fraction by applying a magnetic separator to the mixed solution. The Tsol-p27 was purified in parallel as described previously [45].

### **Polyclonal sera produced in rabbits**

**Paper I:** The recombinant Tsol-p27 protein was expressed as glutathione S-transferase (GST) fusion protein and then purified and used to raise polyclonal antibodies in rabbits as previously described [45], 46].

In short, on days 0, 28, 56, and 84, each of two male rabbits received four immunizations with the recombinant proteins (250 µg/rabbit) emulsified in 250 µl of Freund's complete (first immunization) or incomplete (second to fourth immunization) adjuvant. Sera collected before immunizations were used as negative control. Serum was separated and stored at –20°C until used.

### **Western blot analysis**

**Paper I:** Western blot analysis of anti-rabbit Tsol-p27 immune sera was performed essentially as in our earlier study [44], with the following changes: extracts from adult tapeworms and cysticerci were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Sigma). The blots were washed and incubated for 1 h with the anti-rabbit IgG conjugated with peroxidase (Sigma) and diluted 1:6,000 in 5% skim milk-PBS. After washing, the membranes were stained with 3,3',5,5'-tetramethylbenzidine (TMB; Sigma).

### **Immunohistochemistry**

**Paper I:** The anti-rabbit Tsol-p27 immune sera were also used to localize the Tsol-p27 proteins in *T. solium* cysticerci. The cysticerci used in this study were dissected from the muscles of a naturally infected pig and then washed in PBS (pH 7.5). Only cysticerci that exhibited a vesicular appearance were selected for the study, and they were stored at –20 °C until used. Ribbons of 8-µm sections of the *T. solium* cysticerci were placed on slides, and then treated as follows: air dried for 20 min, fixed in acetone for 20 min, dried for 15 min at room temperature, and thereafter rehydrated in PBS. Anti-rabbit Tsol-p27 or TsolHSP36 immune serum diluted 1:40 in PBS was added to the slides, which were subsequently incubated for 45 min. As a

negative control, pre-immune sera were used at the same dilution and incubation time. After the incubation, the slides were washed 2 x 15 min in PBS and then incubated for 45 min with fluorescein isothiocyanate (FITC)-labelled anti-rabbit IgG (Sigma) diluted 1:40 in Evans blue solution. Lastly, the slides were washed 2 x 15 min and examined in a fluorescence microscope (Leica Microsystems).

### **Amino acid sequencing analysis**

**Paper I:** Sequencing analysis of Tsol-p27 was performed at the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) using the BLAST search option with a cut off of  $10^{-5}$ . To obtain the family domain description corresponding to Tsol-p27, a cut-off of  $10^{-9}$  was employed in the search option.

### **Ethics**

The use of human samples was approved by the Ethics Committee of the Faculty of Medicine of Eduardo Mondlane University and the National Committee on Biomedical Research Ethics of the Ministry of Health of Mozambique.

## 9 RESULTS

### **Paper I: Identification of immunoreactive proteins from *T. solium***

Soluble antigens from *T. solium* cysticerci were separated according to isoelectric point (*pI*, range 4–7) and size (MW), and were thereafter visualized by Coomassie Blue staining (Fig. 3) and transferred to nitrocellulose membranes. The membranes were then probed with sera from NCC-positive or NCC-negative patients (Fig. 3). Proteins that reacted with NCC-positive but not NCC-negative sera were cut out of the Coomassie-stained gel and subjected to partial amino acid sequencing.

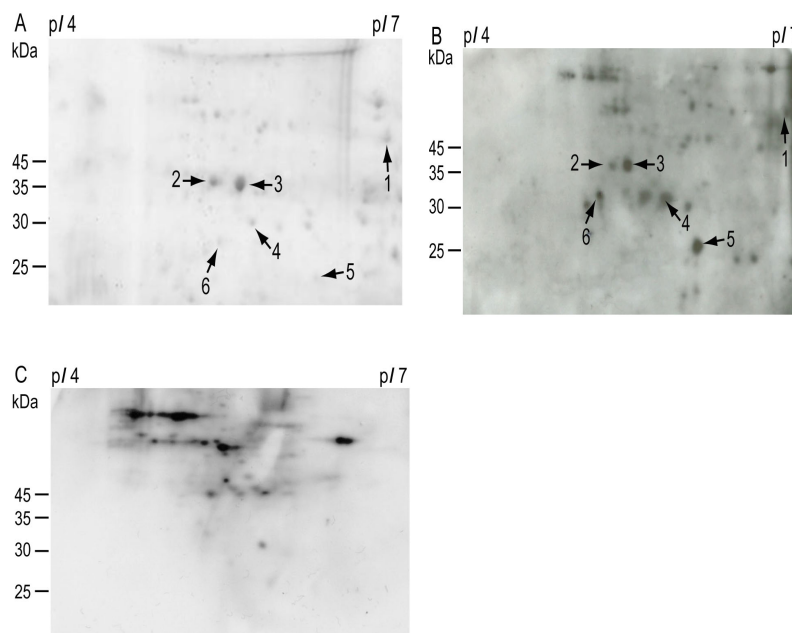


Figure 3. 2-DE Western blot analysis of *T. solium* cysticerci. (A) A 2-DE gel stained with Coomassie Blue showing soluble cysticerci proteins with *pI* values in the range 4–7. (B) Corresponding Western blot probed with serum from a patient diagnosed with NCC. (C) Corresponding Western blot probed with serum from a control subject. Arrows indicate the proteins that were isolated and sequenced.

Six proteins cut out of the gel were subjected to LC–MSM analysis. The amino acid sequences from several peptides, derived from each of the selected proteins, were determined (Table 1). The peptide sequences were identified as possible proteins, determined by the degree of homology to other known proteins. The following proteins were identified: tropomyosin, enolase related to *T. asiatica*, cC1 antigen, actin, heat shock protein p36, and Tsol-p27 related to *T. solium* (Table 1).

Table 1. Identified proteins recognized by NCC-positive human sera

Spot no.	Protein identity	GenBank accession/Organism	Amino acid sequence	MW (kDa)	pI
1	Tropomyosin	ABN14948.1/ <i>T. asiatica</i>	(K)AINLENQLK(E) (K)NIQTEVDTVQESLQEAISK(L) (R)ATNAEAEVAAMTR(R) (R)LLEEDFEQSSGR(L) (R)MAQLEEQVK(E) (K)YIAEDAER(K) (R)LAVTEVDLER(A) (K)IVELEEEELR(I) (K)SLEVSEQESLQR(E) (R)EESYEETIR(D) (K)LQNEVDRLLEDLLSEK(E)	32.2	4.7
2	Enolase	ACX56268/ <i>T. asiatica</i>	(K)LGANAILGVSLAVCK(A) (K)LAMQEFMILPTGAK(N) SGETEDSTIADIVVGLR (K)HIADLAGNK(N) (K)ACNALLLK(V) (K)YNQLLR(I) (K)VNQIGSVTESIK(A) (R)AAVPSGASTGVHEAVELR(D)	47	6.2

---

			(K)IGMDVASSEFYQDGK(Y)		
			LAEIYLEMLSK		
			IEEELGSK		
3	cC1 antigen	AAD34598.1/ <i>T. solium</i>	NLTPSTLSQVVNPGLAETDAK	37.9	5.7
			EALLLALAGQADEPQAMQLK		
			SFLQLNATNEAYNR		
			YAELYGETLEAAIK		
			ADTDLGSIK		
			DAYPSISSK		
			FALLLIQSPWQVMAEALYDAMK		
			VLNEIIAGCSK		
			SLVHLYAPNGEK		
			TLHDAITSELSGK		
			GDTSGDYEALCLK		
			ASLFAELLHFAMR		
			AFEEVSGGETLDDAIK		
			ELYACGEGRPGTAESR		
4	Actin	AAA30092.1 <i>T. solium</i>	(R)VAPEEHPVLLTEAPLNPK(A)	41.7	5.3
			(R)GYSFTTTAER(E)		
			(K)YPIEHAIVTNWDDMEK(I)		
			(K)QEYDESGPSIVHR(K)		

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			(K)LCYVALDFEQEMATAASSSSLEK(S)		
			(K)IWHHTFYNELR(V)		
			(K)EITSLAPSTMK(I)		
			(K)IVAPPER(K)		
			(K)AGFAGDDAPR(A)		
			(K)DSYVGDEAQSK(R)		
5	Heat shock protein p36	Q7YZT0/ <i>T. solium</i>	(R)NLFSLEPFTAMDNAFESVMK	35.5	5.7
			(R)EFHPELEYTQPGELDFLK(D)		
			(K)SVACGDAAMSESVGR(S)		
			(K)GLAIQPSEVQER(Q)		
			(R)SIPLPPSVDR(N)		
			(K)VYVHGVGTGK(E)		
			(K)EMSAIQPR(E)		
			(K)VYFNVK(N)		
6	Tsol-p27	AEF14021.1/ <i>T. solium</i>	(R)TSDLIHEIDQMK(A)	27	5.0
			(K)IITATEEFVDINIASK(V)		
			(K)LGTALEQVASQSEK(A)		
			(K)NFLNTTLSEAQK(A)		
			(K)EFDGLSVQLLDLIR(A)		
			(K)NYYEACAK(E)		

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Three of the identified proteins were excluded from further investigations because they were extremely conserved and were also found in other parasitic worms and in humans. TsolHSP36 and Tsol-p27 were also detected in a previous study in Nicaragua [44], which suggested that Tsol-p27 would be a suitable candidate for diagnosis of NCC. The third protein chosen to be evaluated and expressed as a recombinant protein in the present investigation was cC1. Purified Tsol-p27 on Western blot strips was recognized by antibodies in all serum samples from the NCC-positive control group (Fig. 4), whereas purified cC1 on such strips was detected by antibodies present in only 50% of the the NCC-positive sera (Fig. 4); neither of those recombinant proteins reacted with any antibodies present in the sera from the healthy control group.

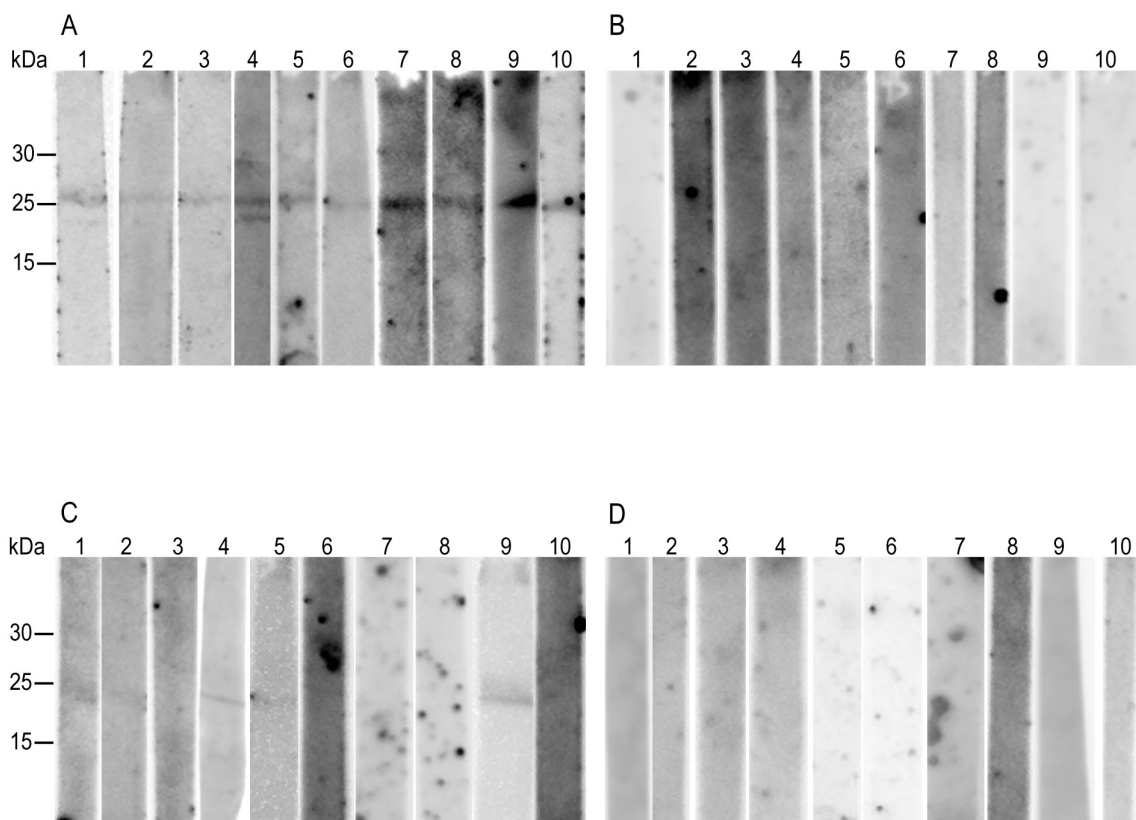


Figure 4. Immunoblot analysis of recombinant Tsol-p27 (A and B) and cC1 (C and D) antigens. (A and C) Probing done with serum (diluted 1:500) from patients

diagnosed as NCC positive by CT (lanes 1–4). (B and D) Probing done with serum (diluted 1:500) from patients diagnosed as NCC negative by CT.

### **Paper I: Localization of polyclonal antibodies raised against Tsol-p27**

To determine the specificity of antibodies raised against Tsol-p27, Western blot analyses were carried out using extracts of cysticerci and adult tapeworms. The size of the protein was predicted to be 27 kDa. The Western blotting of both the cysticercus and adult stage extracts detected the Tsol-p27 protein as a single band of 27 kDa (Fig. 5A, C). Pre-immune serum was used as a negative control (Fig. 5B, D).

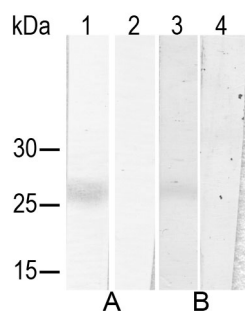


Figure 5. Western blot analysis of extracts of *T. solium* cysticerci (A) and adult tapeworms (B) using anti-rabbit Tsol-p27 immune serum (lanes 1 and 3) and pre-immune serum as negative control (lanes 2 and 4).

Immunofluorescence detection of antibodies raised against Tsol-p27 antigen was used to localize the proteins in *T. solium* cysticercus tissue. As shown in Figure 6, the reaction of antibodies to the protein was seen as intense green fluorescence over the parenchymal folds and the tegument of the spiral canal in *T. solium* cysticerci, whereas no fluorescent reaction was observed when pre-immune serum was tested.

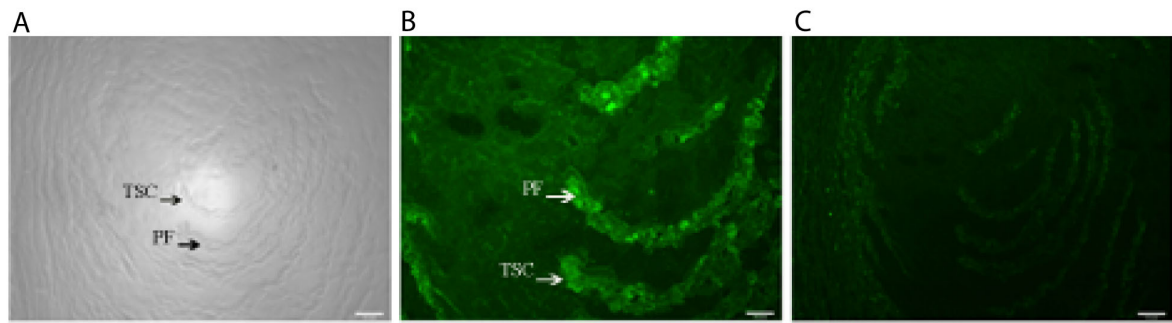


Figure 6. Sections of *Taenia solium* cysticerci examined by phase contrast microscopy and FITC staining. The arrows in panels A and B indicate the parenchymal folds (PF) and tegument of the spiral canal (TSC). (B) An image obtained using rabbit anti-Tsol-p27 antibodies, and structures are indicated as in panels A and B. (C) Image showing the results obtained when using pre-immune sera as controls.

**Paper 2: Performance of Tsol-P27 antigen for serology diagnosis of cysticercosis in Mozambique**

A total of 161 serum samples from the Angonia District and Beira City in central Mozambique were investigated. Fourteen of the samples were positive and 147 negative for Tsol-p27 anti-Tsol-p27 antibodies by EITB assay. Western blotting showed both higher sensitivity and higher specificity compared to the commercial ELISA for analysis of Tsol-p27 (Table 2). Furthermore, the Western blot results were similar to those previously obtained for Tsol-p27 in Central America: 80.00% sensitivity and 96.69% specificity in Mozambique versus 86.7% sensitivity and 97.8% specificity in Nicaragua. If four positive samples from paper 1 are added, the sensitivity increases to 85.71% while the specificity remains the same. By comparison, the ELISA used in Mozambique provided 15.38% sensitivity and 85.14% specificity.

Table 2. Detection of antibodies against *T. solium* cysticerci in serum samples by two different immunodiagnostic tests compared with the gold standard method EITB.

\*The number of positive samples when samples from Paper 1 are added.

	EITB		
	Positive	Negative	Total
<b>Commercial ELISA</b>			
Positive	2	22	24
Negative	11	126	137
Total	13	92	161
<b>Western blot</b>			
Positive	8 (12)*	5	13(17)*
Negative	2	146	148
Total	10 (14)*	151	161

## 10 DISCUSSION

Immunodiagnosis of human cysticercosis has been accomplished using a variety of antigens and methods, including ELISA, dot blot, and EITB [47-50]. Among the tests that have been developed, EITB is considered to be the most specific and is therefore also regarded as the gold standard. This technique uses an enriched fraction of glycoproteins prepared from cysticerci by lectin affinity purification [48]. However, the main drawbacks of EITB are the cost and the reproducibility when investigating serum samples from patients carrying only one or a few cysts. Therefore, research projects have been focused on developing new serological diagnostic tools that offer sensitivity and specificity, and are easy to perform in poorly equipped laboratories. The aim of the present project was to improve the diagnosis of cysticercosis in rural settings.

Here, the protein Tsol-p27 was addressed as a promising novel candidate for future use in diagnosis of NCC. In our work, the Tsol-p27 antigen was efficiently expressed in *E. coli* as a fusion protein and was purified in two steps using glutathione-agarose and his-selected nickel beads, respectively. We found that Western blotting was comparable to commercial ELISA for analysis of Tsol-p27, with regard to both specificity and sensitivity. In previous research conducted by our team in [45], it was noted that the Tsol-p27 antigen showed potential for diagnosis of human cysticercosis. Similar to those results, the current Western blot analyses based on a population in Mozambique that the Tsol-p27 antigen was identified by antibodies in serum from NCC patients, but not by antibodies in serum from a control group. There is evidence that using ELISA or other immune diagnostic methods to detect specific antibodies against *T. solium* in serum can be a useful tool for identifying cysticercosis in humans. However, the commercial ELISA we used provided very poor results, that is, it identified only two of 14 patients that were positive for NCC by EITB assay. An explanation for this low sensitivity might be that the serum samples that were analysed came from patients carrying very few cysts, or even that the ELISA applied used antigens from pigs infected with *T. solium*. Such porcine-derived material can vary in quality, and in our study might have represented a batch of proteins that were less immune reactive or were partially degraded during the production process. Indeed, the *T. solium* antigens currently used for diagnosis of human cysticercosis are derived from parasites extracted from infected pork [48]. It is plausible that recombinant antigens can serve as a simplified, low-cost substitute

for the native antigens, and also provide a reproducible diagnostic assay that can be employed in the field.

## **11 CONCLUDING REMARKS**

**Paper I:** In this study, Tsol-p27 and cC1 were identified, and serum samples were evaluated. Although the number of samples assessed was limited, Tsol-p27 was identified as a potential candidate for diagnosis of cysticercosis. Therefore, it can be recommended that a larger number of samples be evaluated. The function of Tsol-p27 might be related to a variety of biological processes that involve membrane sorting, fusion, and transport. Furthermore, this protein might aid and promote the growth of the adult tapeworm within the human intestine.

**Paper II:** For evaluation of Tsol-p27, the Western blot analysis performed well in comparison with the other diagnostic tests that were assessed. Importantly, Western blotting is simpler to use and less expensive than ELISA and EITB, and it would be relatively easy to implement the Western blot Tsol-p27 assay in poorly equipped laboratories in low-income countries.

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