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The highly antigenic 53/25 kDa *Taenia solium* protein fraction with cathepsin-L like activity is present in the oncosphere/ cysticercus and induces non-protective IgG antibodies in pigs

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Abstract

Cathepsin L-like proteases are secreted by several parasites including *Taenia solium*. The mechanism used by *T. solium* oncospheres to degrade and penetrate the intestine and infect the host is incompletely understood. It is assumed that intestinal degradation is driven by the proteolytic activity of enzymes secreted by the oncosphere. Blocking the proteolytic activity by an antibody response would prevent the oncosphere penetration and further infection. Serine and cysteine proteases including chymotrypsin, trypsin, elastase, and cathepsin L, are secreted by *T. solium* and *Taenia saginata* oncospheres when cultured in vitro, being potential vaccine candidates. However, the purification of a sufficient quantity of proteases secreted by oncospheres to conduct a vaccine trial is costly and lengthy.

A 53/25 kDa cathepsin L-like fraction partially purified from *T. solium* cyst fluid was described previously as an important antigen for immunodiagnostics. In this study we found that this antigen is present in the *T. solium* oncosphere and is also secreted by the cysticercus. This protein fraction was tested for its ability to protect pigs against an oral challenge with *T. solium* oncospheres in a vaccine trial. IgG antibodies against the 53/25 kDa cathepsin L-like protein fraction were elicited in the vaccinated animals but did not confer protection.

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Conflict of interest statement

All authors disclose any conflict of interest

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Keywords

cathepsin L; cysticercosis; oncosphere; protease; *Taenia solium*; vaccine

Introduction

Taeniasis and cysticercosis are parasitic diseases caused by different life stages of the tapeworm *T. solium*. Taeniasis (infection with the adult tapeworm) is exclusive to humans and results from the ingestion of larval cysts. Cysticercosis (infection with the larval stage of the parasite) resulting from the ingestion of eggs usually occurs in pigs, the intermediate host. Humans may also act as an intermediate host and develop cysticercosis after the accidental ingestion of eggs through contaminated food or fecal oral transmission. Cystic larvae often locate in the central nervous system, causing the clinical disorder known as neurocysticercosis (NCC), a major global cause of acquired epilepsy (Carpio and Hauser, 2002; Garcia and Del Brutto, 2000; Nicoletti et al., 2002) that is associated with significant neurological morbidity in developing countries (White and Garcia, 1999). Documentation of *Taenia solium* in the US and other developed countries, at least in modern times, has almost entirely involved “imported cases” from foreign endemic regions (Marconi et al., 2006; Schantz et al., 1992; Tsang V and M, 1995; White, 1997). Despite several recent investigations using mass chemotherapy alone to treat populations for this infection, no sustainable eradication has yet been achieved (Garcia et al., 2007; Garcia et al., 2006; Gonzalez et al., 1998). Porcine vaccines are promising tools given the essential role of the pig as obligatory intermediate host. Such a tool is indispensable to achieving sustained control of this disease. Porcine vaccination strategies to control this disease have been conducted in multiple studies by several groups (Gauci et al., 1998; Lightowlers, 2003; Lightowlers et al., 2003; Nascimento et al., 1995; Pathak and Gaur, 1990).

Although not demonstrated yet, it is assumed that the infection starts with the adherence of the oncosphere to the intestine followed by a proteolytic attack to degrade the intestinal wall and start the penetration. Blocking either the adherence or the proteolytic attack of the oncosphere would prevent infection. Antibodies raised to the specific proteins involved in these processes, as a result of immunization, would confer a desired protection.

Porcine vaccines are promising tools in the control of taeniasis/cysticercosis, given the essential role of the pig as the obligate intermediate host. Vaccines are crucial to achieve sustained control and possible eradication of this disease. Oncosphere antigens are currently the most effective vaccine candidates in preventing porcine cysticercosis (Assana et al.; Flisser et al., 2004; Gonzalez et al., 2005; Lightowlers et al., 2003; Pathak and Gaur, 1990; Plancarte et al., 1999; Verastegui et al., 2002). *T. solium* oncospherical protein TSOL18 is currently the best antigen able to induce near total protection against a *T. solium* proglottid oral challenge in pigs (Lightowlers, 2003). TSOL18 is an oncosphere protein homologue to *T. saginata* HP6. HP6 has been characterized as an adherence protein with a fibronectin type III domain, able to interact with integrins from epithelial cells (Bonay et al., 2002; Ferrer et al., 2007; Parkhouse et al., 2008).

Several proteases including mainly serine proteases (chymotrypsin-like and trypsin-like proteases), but also cysteine proteases (cathepsin L-like proteases) were found to be secreted by *T. solium* oncospheres (Zimic et al., 2007), eventually to be used to degrade the intestinal wall and let the oncosphere penetrate into the circulatory system. Therefore oncosphere proteases are potential vaccine candidates to prevent the intestinal penetration of the oncosphere. The purification of a large amount of oncosphere proteases for a vaccine trial is extremely expensive and lengthy.

In a previous study, we described a 53/25 kDa protein fraction with cathepsin L-like activity (from now on TsCLf), purified from the cyst fluid. TsCLf showed a high sensitivity and specificity for the diagnostics of NCC, in both a Western immunoblot and an ELISA assay (Zimic et al., 2009).

In the present study we tested the presence of the TsCLf in the oncosphere and in the excretory/secretory antigens of the cyst, and tested the TsCLf as a vaccine candidate for porcine cysticercosis.

Materials and methods

Cysticercus fluid and TsCLf purification

Eight pigs naturally infected with porcine cysticercosis, confirmed by tongue test, were selected from an endemic area in the central highlands of Peru (Gonzalez et al., 1990). The animals were sedated with a combination of xylazine and ketamine, and euthanasia was performed using a pentobarbital overdose. The carcasses were dissected and *T. solium* cysts identified. Cyst fluid was recovered by aspiration with a syringe. Approximately 400 mL of cyst fluid was recovered from 4 000 viable cysts and stored at -70°C . To obtain the TsCLf partially purified antigen, the cyst fluid was processed following the procedures previously described (Zimic et al., 2009).

Detection of TsCLf in the excretory/secretory antigens of the cysticercus

Metacestodes of *T. solium* were individually collected from muscle tissue of naturally infected pigs. Cysts were cultivated for 72 hours at 37°C in 5% CO_2 in 12-well plates with cyst medium (buffered RPMI 1640 with antibiotics) as reported (Mahanty et al., 2011). After each 24 hour period, the supernatant of each well containing excretory/secretory (ES) antigens was harvested, collected and replaced. The collected ES antigens were resolved in a SDS-PAGE and revealed by Western immunoblot using pre-immune and post-immune anti TsCLf rabbit sera as previously described (Zimic et al., 2009). Briefly, after SDS-PAGE, the antigen was transferred to nitrocellulose membrane (BIO RAD). Membranes were incubated overnight at 4°C with pre-immune and post-immune rabbit anti-TsCLf sera (1:200). Membranes were washed three times with PBS-0.3% Tween20, incubated with peroxidase-conjugated goat anti-Rabbit IgG (H+L) antibodies diluted 1: 400 (KPL) for 1h at room temperature and developed with 3,3-diaminobenzidine (Sigma) as the substrate.

Additionally, the excretory/secretory antigens from the cysticercus were tested fluorometrically for cysteine protease activity with the substrate Z-Phe-Arg-AMC and inhibition with E64 as previously described (Zimic et al., 2007).

Detection of TsCLf in the oncosphere

The total antigens from activated oncospheres were obtained following the procedure previously reported (Verastegui et al., 2003). Adult *T. solium* tapeworms were collected from newly diagnosed patients. The eggs were obtained from gravid proglottids of adult tapeworms by gentle homogenization in 2.5 % potassium dichromate solution (Sigma, St. Louis, Missouri). With a solution of 0.75% sodium hypochlorite (Mallinckrodt Baker, Inc, Phillipsburg, NJ) in water for 10 min at 4°C , the eggs were hatched and oncospheres were released. Oncospheres were then washed three times in RPMI medium (Sigma). Onospheres were activated by incubation at 37°C for 45 min with artificial intestinal fluid (1 g pancreatin [Sigma Chemical Co., St. Louis, MO], 200 mg Na_2CO_3 , and 1 mL pig bile, with the volume adjusted to 100 mL with RPMI 1640 medium [pH 8.04]) followed by another incubation for 1 hour at 37°C RPMI medium (Verastegui et al., 2007). After activation, the oncospheres were washed three times in phosphate-buffered saline (PBS), pH 7.5. Then the

activated oncospheres were sonicated at 4°C at 70 Hz (Sonic-Dismembrator model 300; Fisher Scientific, Pittsburgh, PA) using three one-minute periods separated by one-minute rest intervals, and centrifuged at 10 000 × g for 30 minute at 4°C and the supernatant were used as total antigens.

The oncosphere total antigens were resolved in a SDS-PAGE and revealed by Western immunoblot using a pre-immune and post-immune rabbit anti-TsCLf sera as described above.

Additionally, TsCLf was resolved in a SDS-PAGE and revealed by Western immunoblot using pre-immune and post-immune rabbit anti-oncosphere sera, as described before (Zimic et al., 2009).

Rabbit anti-TsCLf sera

Three two-month old New Zealand white rabbits, each weighting approximately 2 kg, were immunized with the TsCLf fraction in four doses given at intervals of four days. The first dose was 5 µg of antigen with 50 µg of saponin as adjuvant. The second, third, and fourth doses included 10, 20, and 40 µg of antigen respectively with 50 µg of saponin as adjuvant with each dose. 0.5 mL of blood was sampled on the day prior to each immunization. Two weeks after the last dose, the animals were sacrificed and almost 100 mL of blood was recovered. Pre-immune and post-immune anti-TsCLf sera corresponding to the second and fourth dose were tested in a Western immunoblot using the TsCLf as the antigen according to the conditions described previously (Zimic et al., 2009).

Additionally, two similar rabbits were immunized with total *T. solium* oncosphere antigens prepared as described above. The immunization protocol was similar to the described above. Pre-immune and post-immune anti-oncosphere sera was obtained.

Porcine vaccine trial

Six two-month-old pigs (Pietrain/Landrace mixed breed) were included in this vaccine trial. This study was authorized by the Animals Ethics Committee of the School of Veterinary Medicine, Universidad Nacional Mayor de San Marcos. Each animal was immunized with two doses of purified TsCLf (0.2 mg) given with 1 mg saponin as adjuvant given two weeks apart. Because this study was nested in a larger vaccine trial that tested a second antigen that was fused to Glutathione S-transferase (GST), obtained from the TOPO vector (Invitrogen). The control/placebo group included six similar pigs that received two doses of recombinant GST (0.5 mg, expressed from the TOPO vector), with 1 mg Quil A as adjuvant (Superflos Biosector, Vedbaek, Denmark) given two weeks apart. It has been proved that GST does not protect pigs against cysticercosis infection (Flisser et al., 2004), therefore its administration to the placebo group is not critical. Blood samples were obtained before immunization and one week after the first and second doses. Sera were tested for anti-TsCLf IgG antibodies in a Western immunoblot. Other study testing Tsol18 showed that at 2 weeks after the second dose, antibodies are able to elicit strong immunity or confer protection against cysticercosis in pigs (Assana et al.; Jeri et al., 2004; Verastegui et al., 2003). Flisser et al. tested Tsol18 and Tsol45 with an infection at 3 weeks after the last dose (Flisser et al., 2004). A similar study confirmed protection of this vaccine with an infection at 10 days after the last dose (Gonzalez et al., 2005). After confirming that anti-TsCLf IgG antibodies were elicited and strongly reacted in a Western immunoblot, animals were challenged with one gravid proglottid given orally (Jeri et al., 2004; Verastegui et al., 2003) 15 days after the last dose, to ensure an appropriate immune response. Five different tapeworms were used for the challenge.

Twelve weeks after challenge, all animals were humanely euthanized and the entire carcass and brain were assessed for the presence of cysticerci (Gonzalez et al., 2005; Verastegui et al., 2002). Cysts were classified as either viable, if a defined cystic structure with a liquid content was still present, or degenerated, if this had been replaced by semisolid contents or inflammatory scar tissue (Gonzalez et al., 2005).

Statistical analysis

The total number of cysts as well as the number of cysts of each category (i.e. viable and degenerated) was compared between the control and the vaccinated groups. To adjust for potential confounding, the comparison was performed using a multiple Poisson regression after adjusting for the sex and age of the pig, as well as the tapeworm identity because proglottids from different tapeworms were used for the challenge (proglottids from different tapeworms may have different infectivity). An indicator variable referring to the vaccine group was used to test the vaccine efficacy.

Results

Detection of TsCLf in *T. solium* oncosphere and cysticercus excretory/secretory antigens

The oncosphere total antigens, the cysticercus ES antigens, and the purified TsCLf fraction reacted positively in the Western immunoblot against rabbit anti-TsCLf sera. Reactive bands at 53 kDa and 25 kDa were detected. No reaction was observed against the pre-immune rabbit sera (figure 1). The ES antigens from the cysticercus showed a positive cathepsin L-like activity by being able to hydrolyze the Z-Phe-Arg-AMC substrate, and was inhibited by E64. A weak band appeared between 25 and 53kDa. This was previously described as a contamination of the partial purification (Zimic et al., 2009). This band is not associated to a proteolytic activity and is not sensible/specific for cysticercosis.

Porcine vaccine trial

IgG antibodies against the TsCLf were induced in pigs after the second dose of immunization. The pre-immune sera of the six pigs were not reactive against the TsCLf in the Western immunoblot (Figure 3). All vaccinated animals harbored cysts, therefore no evidence of complete protection was observed in any animal. A trend towards reduced numbers of total cysticerci in the vaccinated group (mean= 718 cysts/pig) compared to the control group (mean= 1 025 cysts/pig) was observed (Table 1), although this difference was not statistically significant ($P= 0.3$ for a Poisson regression adjusted for the tapeworm identity). Furthermore, no significant difference was observed in the number of viable and degenerated cysts between the vaccinated and control groups. One vaccinated pig showed only four degenerated cysts and no viable cysts. One taenia-proglottid was not infective, causing one and no cysts in two control animals respectively. The data corresponding to this taenia was not considered in the analysis.

Discussion

The TsCLf was confirmed to be present in the *T. solium* oncosphere and to be secreted by the cysticercus. In a previous study, we demonstrated that the oncosphere antigens were able to hydrolyze the Z-Phe-Arg-AMC substrate, and were inhibited by E64 (Zimic et al., 2007). These results support the current finding of TsCLf in the oncosphere. However the TsCLf did not confer a complete protection in vaccinated pigs against an oral challenge with *T. solium* proglottids. This negative result could be taken as evidence to contribute towards the understanding of the molecular mechanism of *T. solium* oncosphere infection.

In both humans and pigs, oncospheres cross the intestinal lumen and are transported throughout the body in the blood stream depositing most often in muscle, eyes, and brain tissue. The mechanism used by the oncospheres to penetrate the intestine is still unknown; however, it may involve a two-stage process starting with adherence to the intestinal epithelium followed by degradation of the intestinal wall driven by oncosphere-secreted proteolytic enzymes. The interruption of any of these two events should cause a level of protection against cysticercosis infection. Therefore, proteins involved in either of these two steps may be used as vaccine antigens.

TSOL18 is a *T. solium* oncosphere protein that has been shown repeatedly to be highly protective when used as a vaccine against porcine cysticercosis (Kyngdon et al., 2006; Lightowlers, 2003). The recombinant form of this protein is currently the most important vaccine which confers almost complete protection in pigs (Assana et al., 2010). Proteolytic activity is widely used by many parasites to facilitate tissue penetration and infection. Serine-, cysteine-, metallo- and amino-peptidases are secreted for this purpose (Fukase et al., 1984, 1985; Halawa and Jakacka, 1977; Kong et al., 1994; Marco and Nieto, 1991; McKerrow, 1989; Song and Chappell, 1993; White et al., 1992). In a previous study we found that *T. solium* and *T. saginata* oncospheres secrete several proteases including chymotrypsin-like, trypsin-like, elastase-like and cathepsin L-like proteases (Zimic et al., 2007). In the case of *Fasciola hepatica*, a secreted cathepsin L facilitates the penetration through the tissue of its host and is also an important immunodiagnostic and protective antigen (Collins et al., 2004; Cordova et al., 1997; Cordova et al., 1999; Cornelissen et al., 1999; Harmsen et al., 2004; Tantrawatpan et al., 2005). A study on *Taenia spp.* demonstrated that metacestodes secrete a number of proteolytic enzymes including metallo-aminopeptidases and cysteine proteases that degraded human IgG (Baig et al., 2005). As in several parasites, *Taenia* metacestodes may be using proteases to modulate and assist in evasion of the host immune response. Cathepsin L-like proteases are involved in the pathogenesis of several helminths (Trap and Boireau, 2000). They contribute to the tissue invasion by degradation of the extracellular matrix. Several studies have demonstrated that cathepsin L-like proteases are important and required for tissue invasion, as occurs with the trematode *F. hepatica* (Collins et al., 2004). A *F. hepatica* cathepsin L-like protease has been shown to be an important immunogen to prevent infection in cattle. A probable protective mechanism of cathepsin L-like protease activity in this parasite assumes the immune response can be directed against the pro-enzyme, preventing its maturation (Harmsen et al., 2004).

Previous studies demonstrated the production of IgG antibodies after immunization with the highly protective TSOL18 and it was believed that IgG antibodies are responsible for protection (Assana et al.; Flisser et al., 2004). Since our vaccine trial involved intradermal immunization, a systemic IgG response was expected. However, it is possible that an IgA immune response at the intestinal mucosal level was also elicited. Using proglottids as experimental source of infection is problematic due to the great variability in resulting numbers of cysts (de Aluja et al., 1996; Sciutto et al., 1995). Even proglottids from a single tapeworm differ significantly in numbers, maturity, and viability of the ova. This is clearly illustrated in the data here in which controls varied from 0 and 1 to over 4 000 parasites counted. While this variability precludes accurate assessment of protective efficacy, there were established infections in most controls and cases and thus our main conclusion of absence of complete protection is well supported by the data. The variability of the yield however, precludes us from ruling out the existence of partial protection.

The vaccine trial was nested in a larger study, and the controls received rGST and a different adjuvant. This suboptimal design was chosen given the opportunity raised by a concurrent trial with consequent cost and logistical advantages. Although imperfect, the choice of

controls did not affect the interpretation of the negative result given the lack of total protection observed in the vaccine group. Regardless the outcome in the control group, the finding of infected animals in the vaccinated group determines a lack of total protection of the vaccine. The presence of viable cysts in the vaccinated animals suggests that circulating anti-TsCLf antibodies do not result harmful to the cysticercus after their establishment in the different organs of the pig. This result is consistent with our recent finding, which showed that TsCLf is secreted by the cysticercus during infection in pig brain, and it is located on the internal surface of the cysticercus membrane (to be published elsewhere).

The lack of protection of TsCLf suggests that this protease is not likely to be involved in the intestinal wall degradation. Given the basic pH of the intestine environment and given that oncosphere secretes majorly serine proteases (Zimic et al., 2007), it is likely that the penetration of the intestine should be driven by a serine protease rather than a cysteine protease such as the TsCLf tested in this study.

In conclusion, TsCLf was confirmed to be present in the oncosphere and secreted by the cysticercus and was not able to confer protection for porcine cysticercosis.

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Figure 1. Presence of the 53/25 kDa cathepsin L-like purified fraction in the oncosphere and the excretory/secretory antigens of the cyst

(A) Western immunoblot of the oncosphere total antigens revealed with anti-TsCLf rabbit sera, (a) pre-immune sera, (b) post-immune sera; (B) Western immunoblot of the excretory/secretory cysticercus antigens revealed with anti-TsCLf rabbit sera, (a) pre-immune sera, (b) post-immune sera; (C) Western immunoblot of the purified TsCLf revealed with anti-oncosphere rabbit sera, (a) pre-immune sera, (b) post-immune sera.

Figure 2. Antibody response elicited in a rabbit immunized with the 53/25 kDa cathepsin L-like purified fraction in a Western immunoblot assay

Pattern of the Western immunoblot of the purified 53/25 kDa cathepsin L-like fraction revealed against the pre-immune and the post-immune (fourth dose) anti-TsCLf rabbit sera. (+) Positive control and (-) negative control, revealed against a positive and negative cysticercosis human sera. The reactive bands at 53, 25, 10 and 7.5 kDa match with what was previously reported (Zimic et al., 2009).

Figure 3. Antibody response elicited in pigs immunized with the 53/25 kDa cathepsin L-like purified fraction in a Western blot assay

Pattern of the Western immunoblot revealed against the pre-immune and post-immune sera (sera after the second dose) before the challenge of the immunized pigs. (+) Positive control revealed against a cysticercosis-positive human sera.

Table 1

Numbers of *Taenia solium* cysticerci in control and vaccinated pigs

Group	Tapeworm ID used for challenge	Cyst Count		Mean of total cysts [95% Conf. Interval]
		Viable	Degenerated	
Control				
rGST	4	0	0	0
rGST	1	4 092	36	4 128
rGST	5	32	878	910
				732.7(1 025) *
				[0.2 151]([0.3 230]) *
rGST	4	0	1	1
rGST	3	0	33	33
rGST	1	0	22	22
rGST	2	2	33	35
Vaccinated				
Cathepsin L	2	17	76	93
Cathepsin L	5	0	4	4
				599(718) *
Cathepsin L	1	81	25	106
Cathepsin L	5	973	163	1 136
Cathepsin L	4	0	1	1
Cathepsin L	3	2 196	58	2 254
				[0.1561]([0.1930]) *

* statistics calculated after removing the data of the three pigs challenged with tapeworm #4.