

## Effective Production and Purification of the Glycosylated TSOL18 Antigen, Which Is Protective against Pig Cysticercosis<sup>∇</sup>

Xuepeng Cai,<sup>1†\*</sup> Gailing Yuan,<sup>1†\*</sup> Yadong Zheng,<sup>1†</sup> Xuenong Luo,<sup>1</sup> Shaohua Zhang,<sup>1</sup> Juntao Ding,<sup>1</sup> Zhizhong Jing,<sup>1</sup> and Chengping Lu<sup>2</sup>

Key Laboratory of Zoonoses of CAAS, Key Laboratory of Veterinary Parasitology of Gansu Province, State Key Laboratory of Veterinary Etiological Biology, Lanzhou Veterinary Research Institute, CAAS, Lanzhou 730046, Gansu,<sup>1</sup> and Nanjing Agricultural University, Weigang 210095, Nanjing,<sup>2</sup> China

Received 27 March 2007/Returned for modification 9 May 2007/Accepted 16 November 2007

**Cysticercosis caused by *Taenia solium* metacestodes is a worldwide public health problem. Important progress in the development of effective and practical vaccines against this disease has been made. In this study, the promising *T. solium* oncospherical vaccine candidate named TSOL18 antigen was produced in a 5-liter fermentor. During the process of fermentation, the pH of the culture was always kept below 5.0, and in order to prevent foaming, an antifoam agent was added. In addition, the oxygen content of the culture was constantly kept at >50% in our experiment. A high level of the glycosylated protein (2.5 g/liter) was obtained, and the protein was easily purified by gel chromatography. Vaccination trials showed that the recombinant TSOL18 antigen induced 94 and 100% reductions in metacestode burdens in vaccinated pigs, obviously higher than the 89% reduction in pigs immunized with cysticercus crude extracts in trial 1. These are very promising results in the development of an efficient tool to control cysticercosis in Asia.**

Larval cysts of *Taenia solium* are important parasites that cause neurocysticercosis when larvae encyst in the human central nervous system. The larvae can develop into tapeworms in the intestine when people eat uncooked or poorly cooked cysticercotic pork. The tapeworm carrier as the definitive host is the source of *T. solium* eggs, which are released into the environment with the feces. *T. solium* oncospheres can develop into the larval stage in pigs or humans after the ingestion of the released eggs. Cysticercosis is a serious public health problem that affects human beings in many developing areas worldwide, and it has been reported recently in some developed countries (19).

Previous animal vaccination experiments using defined proteins, synthetic peptides, recombinant phages, and plasmid DNA have resulted in high-level efficacy of protection against experimental *T. solium* infection (1, 7, 9, 10, 16, 21). A gene encoding TSOL18 antigen, a homologue of *T. ovis* To18 (8) and *T. saginata* TSA18 (14), which induce protective responses in immunized sheep and cattle, respectively, was screened from a cDNA expression library for *T. solium* oncospheres (4). Recent studies demonstrated that most of the TSOL18-vaccinated pigs were protected against experimental infection (2). In our laboratory, major limitations on the mass production of TSOL18, for instance, a relatively low expression level, instability, and difficulties in purification on a large scale, were found. Thus, a eukaryotic expression system based on *Pichia pastoris* for the production of glycosylated TSOL18 was tested,

and the antigen was purified and evaluated for the development of an effective vaccine against *T. solium*.

### MATERIALS AND METHODS

**Pigs and *T. solium* eggs.** Fifteen 40-day-old healthy pigs, in which anticysticercosis antibodies were not detected by an enzyme-linked immunosorbent assay (ELISA), were purchased from a local area without the occurrence of cysticercosis. The experimental protocol was approved by the Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Lanzhou, China.

An adult worm recovered from a taeniasis patient was completely scissored to release *T. solium* eggs. After being washed three times in saline, the released eggs were recovered by centrifugation, counted under a microscope with a McMaster egg counter, and stored in RPMI 1640 medium at 37°C overnight. The viability of the eggs was checked mainly by their morphology.

**Preparation and purification of recombinant TSOL18 produced in *P. pastoris*.** Recombinant *P. pastoris* cells were constructed previously in our laboratory. Recombinant TSOL18 antigen was produced in a 5-liter bioreactor (B. Braun Biotech). During the fermentation, the pH of the culture was always kept below 5.0, and in order to prevent foaming, Antifoam 204 (Sigma) was added. Furthermore, the oxygen content in the culture was constantly kept at >50% in our experiment. All procedures and other parameters adhered strictly to the instructions in the manufacturers' manuals. The recombinant protein was purified by Sephadex G-100 gel chromatography, eluting with 0.01 M phosphate-buffered saline (PBS; pH 7.2) at 0.5 ml per min. Then the purity level and the concentration of the purified TSOL18 were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a Biophotometer (Eppendorf).

**Analysis of glycosylation.** Twenty microliters of purified TSOL18 was first boiled for denaturation, incubated with endoglycosidase H (BioLabs) at 37°C for 1 h, and then analyzed by SDS-PAGE.

**Preparation of crude antigens from fresh metacestodes.** Crude extracts from *T. solium* cysticerci were prepared by the procedure previously described (13), with a few modifications. Briefly, cysticerci from the skeletal muscle of highly infected pigs were dissected and homogenized in ice-cold saline solution by using a glass homogenizer. The suspension was left overnight at 4°C with gentle stirring, followed by centrifugation for 30 min at 12,000 × g at 4°C. The supernatant, containing a mixture of soluble antigens, was recovered and filter sterilized. The concentration of the antigens was determined using a Biophotometer (Eppendorf).

**Immunization and experimental infection of pigs.** In trial 1, 15 pigs were randomly separated into three groups of five animals each, regardless of their

\* Corresponding author. Mailing address: Xujiaping 1, Yanchangbu, Lanzhou 730046, Gansu, People's Republic of China. Phone: 86-931-8342716. Fax: 86-931-8340977. E-mail for Xuepeng Cai: caixp@public.lz.gs.cn. E-mail for Gailing Yuan: zhyd9@hotmail.com.

† These authors equally contributed to this work.

∇ Published ahead of print on 10 December 2007.

TABLE 1. Detection of specific antibodies in control and TSOL18-immunized pigs in trial 1<sup>a</sup>

| Group (vaccine) | ELISA-measured OD <sub>492</sub> (mean ± SD) |                         |                        |                        |                          |                          |                         |
|-----------------|--|-------------------------|------------------------|------------------------|--------------------------|--------------------------|-------------------------|
|                 | BI   | 20 days pi              | 40 days pi             | 55 days pi             | 70 days pi               | 85 days pi               | 102 days pi             |
| A (PBS)         | 0.13 ± 0.1                                   | 0.15 ± 0.1              | 0.12 ± 0.1             | 0.17 ± 0.02            | 0.21 ± 0.03              | 0.18 ± 0.01              | 0.14 ± 0.1              |
| C (TSOL18)      | 0.13 ± 0.2                                   | 1.01 ± 0.1 <sup>b</sup> | 1.4 ± 0.3 <sup>c</sup> | 1.0 ± 0.1 <sup>b</sup> | 0.81 ± 0.09 <sup>b</sup> | 0.75 ± 0.08 <sup>b</sup> | 0.73 ± 0.1 <sup>b</sup> |

<sup>a</sup> OD<sub>492</sub>, optical density at 492 nm; BI, before immunization; pi, postimmunization.

<sup>b</sup> *P* < 0.05 compared to PBS group.

<sup>c</sup> *P* < 0.01 compared to PBS group.

size, body weight, and sex. The vaccination of pigs with recombinant TSOL18 or crude antigens was performed prior to an experimental challenge infection with *T. solium* eggs. Immunizations were given intramuscularly in the neck. Pigs in group A were given PBS as a control. Group C was immunized with 200 µg of purified TSOL18, and group B was given 1,200 µg of crude antigens extracted from *T. solium* metacystodes. Fifteen days later, a booster dose was administered at the same site. In this animal trial, the adjuvant ISA 206 (Seppic France) was utilized as an immune-response enhancer. Each pig was experimentally infected with 25,000 mature viable *T. solium* eggs 30 days after the booster dose. Control and immunized pigs were slaughtered 90 days after the challenge, and necropsies were performed. The number of encysted cysticerci was determined.

In order to further evaluate the potential use of recombinant TSOL18 for an anticysticercosis vaccine, the vaccination trial with pigs was repeated as described for trial 1 with the exception that the challenge occurred 14 days after the booster dose. As in the previous trial, pigs in group A were given PBS as a control, and group B was immunized with 200 µg of purified TSOL18.

**Detection of specific antibody in pigs in trial 1 by ELISA.** In the first vaccine trial, serum samples from each pig were collected and separated 20, 40, 55, 70, 85, and 102 days postimmunization. Levels of specific antibody against the TSOL18 vaccine or cysticercus crude antigens were detected by an ELISA. Each well of polystyrene plates was coated with 2 µg of the recombinant TSOL18 protein, and the plates were stored overnight at 4°C. The plates were washed three times with PBS–0.05% Tween 20 (PBST) and then blocked with a solution of 4% fetal bovine serum (FBS) in 0.1 M PBST. After incubation for 30 min at 37°C, the plates were entirely emptied and 100 µl of serum (diluted 1:200 in PBST–4% FBS) was dispensed into each well. The plates were incubated for 45 min at 37°C and then washed three times with PBST. One hundred microliters of horseradish peroxidase-conjugated rabbit anti-porcine immunoglobulin G (Beijing Zhongshan Biotechnology) diluted 1:800 in PBST–4% FBS was added, and the plates were incubated for 30 min at 37°C. The plates were washed as described above, 100 µl of diaminobenzidine solution was added, and the mixture in the wells was visualized in the dark at room temperature for 10 min. The optical densities at 492 nm were recorded using an ELISA reader.

**Western blotting.** The proteins were separated on an SDS-PAGE gel and then electrotransferred from the gel onto a polyvinylidene difluoride membrane (Gelman). Western blotting analysis was performed using pig serum obtained 1 month post-experimental infection with oncospheres of *T. solium* (3).

**Statistical analysis.** The significance of the reduction induced by vaccination was determined using the Mann-Whitney test. The antibody levels were compared using an unpaired *t* test.

## RESULTS

**Identification of recombinant TSOL18.** SDS-PAGE results showed that recombinant TSOL18 was secreted into and existed mainly in the supernatant of the culture. The concentration of secreted TSOL18 produced by *P. pastoris* reached 2.5 g per liter. In the supernatant, a recombinant TSOL18 protein was the predominant protein, with only a small amount of the other form of TSOL18, which had a molecular mass of approximately 12 kDa. The recombinant protein was easily separated by gel chromatography, with a high level of purity. After digestion with endoglycosidase H, the 16-kDa component had a decrease in molecular mass of 4 kDa or so, indicating that the 16-kDa protein was glycosylated and the 12-kDa protein was nonglycosylated.

**Specific antibody in experimental pigs in trial 1 in response to vaccination.** Pig sera were collected to detect specific antibody levels throughout trial 1. Four of the five TSOL18-vaccinated pigs showed high levels of anti-TSOL18 antibody, and antibodies reached the highest level 40 days postimmunization (Table 1). One pig in the TSOL18 group failed to exhibit an antibody response, and levels of antibody in serum samples from this pig remained low (optical density at 492 nm, <0.3) over the course of the experiment. High levels of specific antibody in three of the five pigs vaccinated with crude antigens were also detected (data not shown).

**Protection against *T. solium* egg challenge.** Naked-eye inspection was promptly conducted after experimental pigs were humanely killed. Pigs' muscles, brains, and tongues were sliced into small pieces with knives. As shown by the results from trial 1 presented in Table 2, vaccination with the recombinant TSOL18 antigen significantly reduced the number of cysticerci recovered (*P* < 0.01), giving a 94% reduction, 5% higher than the 89% reduction induced by using cysticercus crude extracts. No significant differences between pigs immunized with TSOL18 and those immunized with cysticercus crude antigens were observed. Among three surviving pigs vaccinated with metacystode antigens (two pigs in this group died from unknown causes during the trial), only one was completely protected against experimental infection with *T. solium* eggs. In the TSOL18 group, however, no cysticerci were detected in

TABLE 2. Reduction in numbers of viable metacystodes in pigs immunized with the TSOL18 vaccine or metacystode crude antigens

| Trial and vaccine group (vaccine)     | No. of viable cysticerci in individual pigs | Mean | % Reduction <sup>a</sup> |
|---------------------------------------|---|------|--------------------------|
| Trial 1                               |   |      |                          |
| Group A (PBS) <sup>c</sup>            | 135, 77, 57, 46                             | 78   |                          |
| Group B (crude antigens) <sup>c</sup> | 7, 0, 10                                    | 5.6  | 89 <sup>b</sup>          |
| Group C (TSOL18)                      | 0, 0, 0, 3, 20 <sup>d</sup>                 | 4.6  | 94 <sup>b</sup>          |
| Repeated trial                        |   |      |                          |
| Group A (PBS)                         | 873, 763, 732, 1, 405, 30                   | 761  |                          |
| Group B (TSOL18)                      | 0, 0, 0, 0, 0 <sup>e</sup>                  | 0    | 100 <sup>b</sup>         |

<sup>a</sup> Percent reduction = [1 – (mean number of viable metacystodes in vaccinated group ÷ mean number of viable metacystodes in PBS group) × 100].

<sup>b</sup> *P* < 0.01 compared to PBS group.

<sup>c</sup> One control pig and two pigs immunized with total antigens died during the trial for unknown reasons.

<sup>d</sup> Twenty vesicular cysticerci were detected in one pig with low levels of specific antibody to the TSOL18 vaccine throughout the trial.

<sup>e</sup> Twenty-six calcified cysticerci were detected in a pig immunized with recombinant TSOL18.

three of five pigs. In one TSOL18-immunized pig, low levels of antibodies were elicited and 20 viable encysted metacystodes were detected. Many small calcified cysticerci were detected in individual pigs in the crude-antigen group, but calcified cysticerci were not found in the TSOL18 group (data not shown).

In the repeated trial, no viable cysticerci were detected in group B and all five pigs immunized with recombinant TSOL18 were completely protected from infection with *T. solium* eggs (Table 2). Twenty-six nonviable calcified cysticerci were detected in one of the pigs in group B.

**Immunoblot assay.** Both glycosylated and nonglycosylated TSOL18 proteins produced in *P. pastoris* were recognized by serum from a pig at an early stage of infection with *T. solium* eggs. Not surprisingly, the recombinant deglycosylated antigen also reacted with this serum sample containing anticonosphere immunoglobulin G.

## DISCUSSION

Although several previous studies demonstrated that native *T. solium* oncosphere antigens induce highly protective responses against cysticercosis in pigs (11, 17, 18, 20), vaccines based on such native proteins are impossible in practice because their production is extremely limited. In other animal vaccine trials, defined recombinant oncosphere proteins exhibited a high level of protection against other cestode infections (8, 14, 15). A TSOL18 protein from *T. solium* oncospheres is a very good vaccine candidate and has been successfully tested in vaccination against pig cysticercosis (2). This antigen may be essential for the viability of *T. solium* oncospheres. In vitro experiments reveal that *T. solium* oncospheres coincubated with anti-TSOL18 sera and complement lose viability (12). Recently, Gauci and his colleagues (5) proved that the TSOL18-encoding gene is extremely conserved in 10 *T. solium* isolates from various worldwide geographical locations, which belong to different genotypes on the basis of the *T. solium cox1* gene. This information increases the probability that a TSOL18 vaccine may be useful worldwide to prevent *T. solium* infection in pigs.

The recombinant protein expressed in a glycosylated form in *P. pastoris* closely resembled the native TSOL18 antigen in *T. solium* oncospheres. A high level of expression (up to 2.5 g/liter) was obtained. Moreover, this secreted glycoprotein was easily and efficiently purified from the culture medium by using gel chromatography. The TSOL18 vaccine induced high-level reductions in the parasite load (94 and 100%) in experimental infections with *T. solium* oncospheres in the trials. Considering this information, herein we show the appropriate conditions for the production of this vaccine on a large scale for commercialization.

In this study, high levels of antibodies in four pigs immunized with the TSOL18 antigen in trial 1 were detected by ELISA, but from one pig in the same vaccination group, 20 vesicular cysticercus cysts were recovered. This heterogeneity in the levels of protection induced has also been observed in other vaccination trials (2). No calcified metacystodes were found in any of the pigs vaccinated with the recombinant TSOL18 antigen in trial 1 or in four of five pigs in group B in the repeated trial. The situation was just the opposite for the

trial 1 group immunized with crude antigens derived from *T. solium* larvae. Each of three vaccinated pigs in group B in trial 1 was infested with small, yellow calcified cysticerci. A similar finding was observed in a previous study of pigs vaccinated with DNA based on the gene encoding paramyosin B (6).

In conclusion, herein we describe the procedure for producing the protective TSOL18 glycosylated antigen at high levels. The results described herein also demonstrate for the first time the effectiveness of TSOL18 against the Asiatic *T. solium*.

## ACKNOWLEDGMENTS

This work was supported by the 863 program (2006AA10A207) and a Gansu Key scientific and technological grant (2GS063-A43-013), People's Republic of China.

We are grateful to D. H. Liu for providing the adult worm, to H. Li, Y. X. Dou, D. F. Zhang, H. J. Jia, L. J. Liu, Z. Y. Liu, X. L. Zhu, S. B. Zhao, P. Y. Wang, A. J. Guo, J. L. Hou, Y. Wang, X. Y. Chen, Q. Liu, X. M. Pan, X. X. Wang, F. Yang, and Y. L. He for the dissection of cysticerci from experimental pigs, and particularly to the anonymous reviewers for critical reading and constructive suggestions.

## REFERENCES

- Cai, X., Z. Chai, Z. Jing, P. Wang, X. Luo, J. Chen, Y. Dou, S. Feng, C. Su, and J. Jin. 2001. Studies on the development of DNA vaccine against *Cysticercus cellulosae* infection and its efficacy. *Southeast Asian J. Trop. Med. Public Health* 32(Suppl. 2):105–110.
- Flisser, A., C. G. Gauci, A. Zoli, J. Martinez-Ocana, A. Garza-Rodriguez, J. L. Dominguez-Alpizar, P. Maravilla, R. Rodriguez-Canul, G. Avila, L. Aguilar-Vega, C. Kyngdon, S. Geerts, and M. W. Lightowers. 2004. Induction of protection against porcine cysticercosis by vaccination with recombinant oncosphere antigens. *Infect. Immun.* 72:5292–5297.
- Garcia-Allan, C., N. Martinez, A. Flisser, A. Aluja, J. C. Allan, and P. S. Craig. 1996. Immunocharacterization of *Taenia solium* oncosphere and metacystode antigens. *J. Helminthol.* 70:271–280.
- Gauci, C. G., A. Flisser, and M. W. Lightowers. 1998. A *Taenia solium* oncosphere protein homologous to host-protective *Taenia ovis* and *Taenia saginata* 18 kDa antigens. *Int. J. Parasitol.* 28:757–760.
- Gauci, C. G., A. Ito, and M. W. Lightowers. 2006. Conservation of the vaccine antigen gene, TSOL18, among genetically variant isolates of *Taenia solium*. *Mol. Biochem. Parasitol.* 146:101–104.
- Guo, A., Z. Jin, Y. Zheng, G. Hai, G. Yuan, H. Li, and X. Cai. 2007. Induction of protection against porcine cysticercosis in growing pigs by DNA vaccination. *Vaccine* 25:170–175.
- Guo, Y. J., S. H. Sun, Y. Zhang, Z. H. Chen, K. Y. Wang, L. Huang, S. Zhang, H. Y. Zhang, Q. M. Wang, D. Wu, and W. J. Zhu. 2004. Protection of pigs against *Taenia solium* cysticercosis using recombinant antigen or in combination with DNA vaccine. *Vaccine* 22:3841–3847.
- Harrison, G. B., D. D. Heath, R. P. Dempster, C. Gauci, S. E. Newton, W. G. Cameron, C. M. Robinson, S. B. Lawrence, M. W. Lightowers, and M. D. Rickard. 1996. Identification and cDNA cloning of two novel low molecular weight host-protective antigens from *Taenia ovis* oncospheres. *Int. J. Parasitol.* 26:195–204.
- Huerta, M., A. De Aluja, G. Frago, A. Toledo, N. Villalobos, M. Hernandez, G. Gevorkian, G. Acero, A. Diaz, I. Alvarez, R. Avila, C. Beltran, G. Garcia, J. J. Martinez, C. Larralde, and E. Scuitto. 2001. Synthetic peptide vaccine against *Taenia solium* pig cysticercosis: successful vaccination in a controlled field trial in rural Mexico. *Vaccine* 20:262–266.
- Johnson, K. S., G. B. L. Harrison, M. W. Lightowers, K. L. O'Hoy, W. G. Cogle, R. P. Dempster, S. B. Lawrence, J. G. Vinton, D. D. Heath, and M. D. Rickard. 1989. Vaccination against ovine cysticercosis using a defined recombinant antigen. *Nature* 338:585–587.
- Kumar, V., S. N. S. K. Gaur, and M. L. Pathak. 1993. Immunization of pigs against the cysticercus of *Taenia solium* using fractionated first and second peaks of *Cysticercus cellulosae* scolex antigen. *Indian J. Anim. Sci.* 57:932–935.
- Kyngdon, C. T., C. G. Gauci, R. A. Rolfe, J. C. Velasquez Guzman, M. J. Farfan Salazar, M. R. Verastegui Pimentel, A. E. Gonzalez, H. H. Garcia, R. H. Gilman, R. A. Strugnelli, and M. W. Lightowers. 2006. In vitro oncosphere-killing assays to determine immunity to the larvae of *Taenia pisiformis*, *Taenia ovis*, *Taenia saginata*, and *Taenia solium*. *J. Parasitol.* 92:273–281.
- Larralde, C., R. M. Montoya, E. Scuitto, M. L. Diaz, T. Govezensky, and E. Coltorti. 1989. Deciphering Western blots of tapeworm antigens (*T. solium*, *E. granulosus* and *T. crassiceps*) reacting with sera from neurocysticercosis and hydatidic disease patients. *Am. J. Trop. Med. Hyg.* 40:282–290.

14. Lightowlers, M. W., R. Rolfe, and C. G. Gauci. 1996. *Taenia saginata*: vaccination against cysticercosis in cattle with recombinant oncosphere antigens. *Exp. Parasitol.* **84**:330–338.
15. Lightowlers, M. W., O. Jensen, E. Fernandez, J. A. Iriarte, D. J. Woollard, C. G. Gauci, D. J. Jenkins, and D. D. Heath. 1999. Vaccination trials in Australia and Argentina confirm the effectiveness of the EG95 hydatid vaccine in sheep. *Int. J. Parasitol.* **29**:531–534.
16. Manoutcharian, K., A. Diaz-Orea, G. Gevorkian, G. Fragoso, G. Acero, E. Gonzalez, A. De Aluja, N. Villalobos, E. Gomez-Conde, and E. Sciutto. 2004. Recombinant bacteriophage-based multipeptide vaccine against *Taenia solium* pig cysticercosis. *Vet. Immunol. Immunopathol.* **99**:11–24.
17. Pathak, K. M., and S. N. Gaur. 1990. Immunization of pigs with culture antigens of *Taenia solium*. *Vet. Parasitol.* **34**:353–356.
18. Plancarte, A., A. Flisser, C. G. Gauci, and M. W. Lightowlers. 1999. Vaccination against *Taenia solium* cysticercosis in pigs using native and recombinant oncosphere antigens. *Int. J. Parasitol.* **29**:643–647.
19. Sciutto, E., G. Fragoso, A. Fleury, J. P. Laclette, J. Sotelo, A. Aluja, L. Vargas, and C. Larralde. 2000. *Taenia solium* disease in humans and pigs: an ancient parasitosis disease rooted in developing countries and emerging as a major health problem of global dimensions. *Microbes Infect.* **2**:1875–1890.
20. Verastegui, M., R. H. Gilman, A. Gonzales, H. H. Garcia, C. Gavidia, N. Falcon, T. Bernal, Y. Arana, and V. C. Tsang. 2002. *Taenia solium* oncosphere antigens induce immunity in pigs against experimental cysticercosis. *Vet. Parasitol.* **108**:49–62.
21. Wang, Q. M., S. H. Sun, Z. L. Hu, D. Wu, and Z. C. Wang. 2003. Immune response and protection elicited by DNA immunisation against *Taenia cysticercosis*. *Vaccine* **21**:1672–1680.

---

Editor: W. A. Petri, Jr.