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*Ts*PKA-r: a potential immunodiagnostic antigen for the detection of porcine cysticercosis

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ABSTRACT

Cysticercosis, caused by metacestodes of *Taenia solium*, has a significant soci-economic impact and is of considerable importance in public health. However, there are no specific diagnostic antigens to distinguish between *T. solim* and *Taenia hydatigena*. In the present study, cAMP-dependent protein kinase regulatory subunit (*Ts*PKA-r), an excretory/secretary (ES) antigen of *T. solium*, was used to establish a specific and sensitive diagnostic tool for detection of porcine cysticercosis. The full-length sequence encoding *Ts*PKA-r was amplified by PCR, sequenced and then identified by bioinformatics. The fusion protein with 6 × His-tags was expressed in *E. coli*, purified by Ni Sepharose™ 6 Fast Flow and used to test reactionogenicity by immunoblotting. *Ts*PKA-r based indirect enzyme-linked immunosorbent assays (iELISA) showed good performance in recognition of sera of pigs experimentally infected with *T. solium* metacestodes, with 93.88% sensitivity and 96.40% specificity. There were no cross-reactions against the sera from pigs experimentally infected with *T. hydatigena*, *Toxoplasma gondii* or *Trichinella spiralis*. These results indicate that the *Ts*PKA-r is a promising immunodiagnostic antigen for detection of porcine cysticercosis.

1. Introduction

Taenia solium metacestodes can cause cysticercosis in humans and animals, a neglected disease with a serious human health problem and economic development in developing countries (Flisser et al., 2003; Fleury et al., 2011). Human beings are only a definitive host of *T. solium*, which resides in the small intestine and produces eggs that are shed with the stool. Pigs and humans act as intermediate hosts, which both can get infected by ingesting egg-contaminated food or water (Flisser 1994), causing porcine or human cysticercosis. In some cases, oncospheres can invade the central nervous system and cause neurocysticercosis (NCC) (White 2000).

Current research shows that it is necessary to monitor this zoonosis by developing more effective diagnostic tools (Pawlowski et al., 2005). Previously, many immunodiagnostic techniques have been designed based on different crude extracts from *T. solium* metacestodes using enzyme-linked immunoelectrotransfer blot (EITB) or enzyme-linked immunosorbent assays (ELISA) techniques (Atluri et al., 2009; Dorny et al., 2003). Although some rational results have been obtained, EITB is labor intensive and costly, which limits its usage in epidemiological investigation. Additionally, EITB may not effectively differentiate T. solium from other parasites possibly including Taenia hydatigena (Jayashi et al., 2014). Although *T. hydatigena* is a non-zoonotic parasite, its infectious rate is higher than that of *T. solium* in free-ranging pigs, so cross-reactions between *T. solium* and *T. hydatigena* severely interfere with the exact detection of cysticercosis. Therefore, quick sensitive and specific serological assays are urgently needed for the diagnosis of porcine cysticercosis.

From a diagnostic point of view, excretory/secretary proteins (ESPs) are of specific interest. Immunodiagnostic techniques have been established to detect circulating antigens (CAg) of parasites and specific antibodies in porcine serum samples so far. Substantial amounts of CAg are secreted by parasites, releasing into the host's peripheral blood circulation. Therefore, CAg has a potential value in diagnosing active infections (Pouedet et al., 2002). For *T. solium*, a few specific ESPs have been widely studied and successfully applied in antibody-detecting EITBs and ELISAs (Hancock et al., 2004). Currently, 76 ESPs produced by *T. solium* metacestodes were identified by LC–MS/MS (Victor et al.,

Abbreviations: PKA, cAMP-dependent protein kinase; bp, base pair; PCR, polymerase chain reaction; iELISA, indirect enzyme-linked immunosorbent assays; LB, Luria-Bertani; IPTG, isopropyl-β-d-thiogalactoside; BCA, bicinchoninic acid; TMB, 3,3',5,5'-tetramethylbenzidine; ROC, receiver operating characteristics; AUC, area under ROC curve; HR, likelihood rations; TPV, true positive value; TNV, true negative value

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2012). Many of these proteins have been studied due to their potential as diagnostic antigens in antibody detecting assays (Deckers and Dorny 2010). For example, 18 kDa (*Ts*18), one of the *T. solium* 8 kDa antigen families, is regarded as highly suitable for immunodiagnosis of cysticercosis, with high sensitivity and specificity in antibody detection in the ELISA assays (Espíndola et al., 2005).

cAMP-dependent protein kinase (PKA) is participated in the modulation of protein cascade phosphorylation, and plays a vital role in a biochemical process for signal transduction in different developmental stages of many organisms (Wurtz et al., 2011). Using proteomic approaches (Diaz-Masmela et al., 2013; Victor et al., 2012), PKA was identified as a potential antigen of *T. solium*, which might make it a suitable candidate for development of a specific diagnostic assay for porcine cysticercosis. In this study PKA was identified as a novel antigen, which would be suitable for the development of much more sensitive and specific assays for the diagnosis of porcine cysticercosis.

2. Material and methods

2.1. Parasites and serum samples

Healthy pigs of 40–60 days aged without cysticercosis were purchased from a local area in Gaolan, Gansu Province, China, and all pig blood samples were collected as negative control by saphenous bleeding before infection. Fifty pigs were orally infected with 3, 000 eggs of *T. solium* that was collected from Dali, Yunnan Province, China. Blood samples were collected by saphenous bleeding 60 days post-infection (dpi) (pigs were confirmed by necropsy examination). *Trichinella spiralis* positive sera were from six pigs orally infected with 2, 000 infective muscle larvae (ML) at 35 dpi (pigs were confirmed by necropsy examination). The sera against *Toxoplasma gondii* were obtained from six pigs that were inoculated peritoneally with 1, 000 oocysts.*Toxoplasma gondii* infection was confirmed by PCR as described previously (He et al., 2016) at 15 dpi. *T. Hydatigena* metacestode positive sera (six samples) were collected from a naturally infected pigs from local slaughterhouse in Yongdeng, Gansu Province, China. Pigs were provided with non-medicated feed and water adlibitum in the experiment, housed in ventilated pigsty and monitored every day. At the end of experiments, all pigs were humanely sacrificed, and serum samples were collected and stored at −20 °C until use. Meanwhile, parasites were isolated and stored at −80 °C until use in other experiments.

2.2. Identification and bioinformatic analysis of TsPKA-r

The *TsPKA-r* coding sequence was compared to known entries in GenBank using BLASTp (data not shown). The physicochemical properties of the *Ts*PKA-r including theoretical molecular weight, isoelectric points and amino acid (aa) composition were predicted using the ProtParam software (http://web.expasy.org/protparam/). To gain an insight into probable functions, the deduced *Ts*PKA-r amino acid sequence was scanned against amino acid motif entries, SignalP and TMHMM servers (ExPASY Bioinformatics Resource Server; http://www.expasy.org/proteomics). Putative N-glycosylation sites were identified using NetNGly1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). DNAStar (Version 7.1, DNAStar, USA) was used to predict the potential linear B-cell epitopes of *Ts*PKA-r. The potential T cell epitopes were predicted using the online server SYFPEITHI (http://www.syfpeithi.de/bin/MHCServer.dll/EpitopePrediction.htm) (Rammensee et al., 1999).

2.3. Cloning of TsPKA-r

Total RNA was extracted from *T. solium* metacestodes using Trizol reagent (Invitrogen, USA), referring to the manufacturer's protocol. cDNA was synthesized using One-step Reverse Transcription Kit (Clontech) according to the manufacturer's instructions. The full-length coding sequence of *Tspka-r* was amplified by PCR using specific primers 5′- CGGGATCCATGTCAAAGAAGGCTGTTG-3′ and 5′- CCCAAGCT TGGTAATGGCGTAACTCGGG-3′. The reaction for PCR amplification contained 25 μL premix (dNTPs, PCR buffer, DNA polymerase), 1.0 μL cDNA, 1.0 μL 10 μM of each primer, 22 μL ddH_2O. The amplification reaction was conducted as follows: 95 °C for 5 min; 30 cycles of at 94 °C for 40 s, 65 °C for 40 s and 72 °C for 90 s, and a final extension for 10 min at 72 °C. The PCR products were analyzed on a 1% (w/v) agarose gel and purified by Agarose Gel DNA Extraction Kit (Clontech).

Table 1
Characteristic PKA-r sequences in TsPKA-r.

	Position	Amino acid sequence
Signature 1	135–151	VITLGEDGDNFYVIEKG
	257–273	IISQGQEGDEMYFVEDG
Signature 2	175–192	FGELALMYNTPRAATIQA
	299–316	FGELALLTKHPRAASVYA
cAMP/cGMP binding motif	108–227	LFRCLD...QMYEELLNQ
	230–351	ILQSLS...RNIDNYENQ

Table 2
Potential B cell epitopes position and sequences

Number	Position	Potential B cell epitopes sequences
1	22–28	AVLRNRP
2	39–49	FEMKKNQQKEN
3	61–69	PPIRARRRQ
4	74–81	ESYDPEKE
5	87–96	VVHEKTEEQR
6	136–142	ITLGEDG
7	157–163	VKVDGVE
8	256–264	KIISQGQEG
9	275–287	VRITMKKSGSDEE
10	289–295	ELTRIKK

2.4. Construction of recombinant vector and protein expression

The fragment of *Tspka-r* was digested by restriction enzymes (*Bam* HI and *Hind* III). The digested products were ligated into the pET-30a (+) vector that was also digested by the corresponding restriction enzymes. After ligation, the plasmids were transformed into *E.coli* DH5α competent cells, which were then smeared onto plates of Luria-Bertani (LB) agar supplemented with kanamycin (100 μg/mL), and cultured at 37 °C overnight. After about ten hours, 5 single colonies were selected for inoculation in LB liquid medium containing 100 μg/mL kanamycin, and incubated 4 h at 37 °C. Colonies were screened by PCR amplification, and positive colonies were confirmed by sequencing (GENEWIZ™, China). Positive recombinant plasmids termed as pET-30a (+)-*Tspka-r* were transformed into *E.coli* BL21 competent cells. The transformed colonies were cultured in LB liquid medium containing 100 μg/mL kanamycin at 37 °C. When the OD_{600} value reached 0.6-0.8, isopropyl-β-D-thiogalactoside (IPTG) (0.25 mM to 1.5 mM) was added to induce expression of the recombinant pET-30a(+)-*Tspka-r* for 6 h, followed by protein analysis by SDS-PAGE.

2.5. Protein purification and Western Blot

Recombinant proteins were purified using Ni Sepharose™ 6 Fast Flow purification kit according to the manufacturer's instructions (GE, USA). The concentration of purified protein samples was measured with Pierce™ BCA protein assay kit (Thermo Scientific, USA). The recombinant proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with the positive serum against porcine cysticercosis (1:200 dilution), followed by incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-pig IgG (1:10000 dilution,