



Molecular expression and characterization of *Taenia solium* TS14 gene for sensitive detection of porcine cysticercosis

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ABSTRACT

In the present study, molecular expression and characterization of TS14 gene of *Taenia solium* (Indian isolate) was carried out for sero-prevalence study of porcine cysticercosis. The complete open reading frame (ORF) of TS14 gene was amplified by RT-PCR from mRNA isolated from *T. solium* cysticerci (Indian isolate). The amplicon was cloned into the pET-32a(+) expression vector and used to transform *Escherichia coli* BL21 codon(+) cells to produce TS14 antigen. The nucleotide and deduced amino acid sequences of the TS14 were aligned against the related sequences of *T. solium* available in public domain for *in silico* analysis by DNA STAR and MEGA version 4.0 softwares. The nucleotide sequence revealed that the TS14 gene of *T. solium* (Indian isolate) encodes 85 amino acids and its nucleotide sequence had 98.8% to 99.5% sequence homology with that of China, Peru and Mexico isolates. A high-level expression of the recombinant protein was observed in the molecular range (Mr) of 29 kDa. The rTS14 was confirmed by its specific immunoreactivity against hyper-immune and known reference positive pig sera (8). The protein was serologically non-reactive to known cysticercus negative pig sera samples and further lacks cross-reactivity against hydatid cyst positive pig sera. The present preliminary investigation revealed the good potential of rTS14 for serodiagnosis of *T. solium* cysticercosis.

Key words: Cloning, *Cysticercus cellulosae*, Expression, Pig, *Taenia solium*, TS14

Cysticercus cellulose is a devastating zoonotic disease circulating between humans and pigs. Humans being the definitive host of *Taenia solium*, harbours sexually mature worms in the small intestine, however, development of the cystic stage is not uncommon. Autoinfection with *T. solium* eggs results in establishment of the cystic metacestode in different tissues, brain being the most common. Such a condition is known as neurocysticercosis (NCC) and is manifested as serious neurological symptoms (Garcia *et al.* 1993, White *et al.* 1997). Pigs acquire infection through feed and drinking water contaminated with human excreta containing *T. solium* eggs. In pigs, the larval stage, i.e. cysticercus develops mainly in the heart, brain, muscles of neck, shoulder, thigh, and tongue (Kumar and Gaur 1994). Furthermore, in heavy infections, cysts are observed in liver, lungs, inter-coastal muscles and in ocular tissues (Kumar

et al. 1991, Prasad *et al.* 2002). The infection in pigs is not only responsible for huge economic losses due to carcass condemnation (Flisser *et al.* 2006, Deckers *et al.* 2010), but is also a subject of prime public health concern, particularly for the developing countries like Latin America, Africa and Asia (Geerts *et al.* 2002, Ito *et al.* 2002). Cysticercosis, as an emerging disease, is drawing attention of the developed nations largely due to increased immigration of infected individuals from endemic regions (Sorvillo *et al.* 2007). *T. solium* and cysticercosis are widely prevalent in India and cases from human and swine populations have been documented throughout the country (Varma *et al.* 1989, D'Souza *et al.* 1999, Prasad *et al.* 2002, Sharma *et al.* 2004, Saravanan *et al.* 2014).

An early and accurate diagnosis of porcine cysticercosis is crucial for the identification of endemic regions and can contribute significantly towards the control strategies. Development of a specific and sensitive diagnostic test for porcine cysticercosis would be a major advancement over the traditional less sensitive meat inspection based detection system. Establishing the sero-prevalence of cysticercosis through the detection of anti-parasitic antibodies using a high throughput serodiagnostic test will be a valuable epidemiological tool particularly for large scale mapping of cysticercosis in pigs (Harrison *et al.* 1989, Lightowers

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et al. 1990). Although parasitic cyst fluid derived purified glycoproteins are promising candidates for a rapid diagnosis of infection, however, supply of the native glycoproteins in abundant quantities is a major constraint (Deckers *et al.* 2010, Cai *et al.* 2006). To overcome this constraint, heterologous expression of the target proteins is used to ensure easy availability for diagnostic applications.

TS14, a low molecular weight constituent glycoprotein of *T. solium* cysticerci, is used for serodiagnosis of *T. solium* cysticercus infection in humans and pigs (Tsang *et al.* 1991, Greene *et al.* 2000, Obregon-Henao *et al.* 2003, Assana *et al.* 2007). The protein, a member of the 8 kDa family of *Taenia* antigens, is also known for non-cross reactivity with *Echinococcus granulosus*, *E. multilocularis*, *Schistosoma*, *Trichinella* and *Trypanosoma cruzi* infected sera (Greene *et al.* 2000, Hancock *et al.* 2003, Scheel *et al.* 2005, da Silva *et al.* 2006).

Although TS14 of *T. solium* cysticerci has been studied for its diagnostic potential in East Asian and Mexican countries, Indian isolate derived TS14 gene remains largely unexplored. To this end, the present work describes cloning, expression and preliminary characterization of the TS14 gene from the Indian (Bareilly, Uttar Pradesh) isolate of *T. solium* cysticerci.

MATERIALS AND METHODS

Parasite and reference pig serum samples: Measly pork was collected from pigs immediately after slaughter from Bareilly region and transferred to laboratory for immediate processing. The *T. solium* cysts were dissected out from muscles carefully, washed in ice cold PBS and used immediately for extraction of total RNA. Blood samples from pigs positive for *T. solium* cysticerci infection (8), positive for hydatidosis (1) and negative for *Cysticercus cellulosae* (control group, 2) were collected immediately after slaughter and serum was harvested and stored at -20 °C for further use.

Isolation of total RNA and complementary DNA (cDNA) synthesis by reverse transcription: Total RNA was extracted from *T. solium* cysts using Trizol reagent following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from the total RNA using oligo(dT)18 primer following standard protocol.

Oligonucleotide primers: A pair of PCR primers specific for 258 bp coding sequence of TS14 was custom designed (bold in primer sequences) from a published sequence (Accession No: AF082829.2), incorporating *Nco* I and *Hind* III restriction sites (underlined in primer sequences) in the forward and the reverse primers, respectively, to facilitate directional cloning with expression vector. The nucleotide sequence of the forward and the reverse primers were TS14FS 5' **CATGCCATGGGAATGCGTGCC**TACATTG **TGCTTC** 3' and TS14RS 5' AGTTAAGCTTTTAAGCAG **TTTTTTTCTTAGGACC** 3', respectively.

Polymerase chain reaction (PCR) based amplification of TS14 gene of *T. solium*: The PCR conditions were laboratory standardized for amplification of TS14 coding

sequence of *T. solium* (Indian isolate) in 25 µl reaction volume containing 70 ng of cDNA, 10 pmol each of forward and reverse primers (TS14FS and TS14RS), 1.5 mM MgCl₂, 200 µM of each dNTPs, and 1U of True start Hotstart *Taq* DNA polymerase. The amplification was performed on a thermo cycler with a preheated lid. The cycling conditions were set as an initial denaturation of strands for 2 min at 95°C, followed by 35 repeat cycles of denaturation at 95 °C for 30 sec, annealing of primers at 56°C for 45 sec and extension of strands at 72 °C for 60 sec. A final extension of the synthesized strands was given at 72 °C for 10 min. The PCR amplification was confirmed by running the product on an ethidium bromide stained 1.4% agarose gel and visualization of the amplicon on a transilluminator under UV light.

Molecular cloning and characterization of TS14: The 280 bp PCR amplified product was purified and eluted from the agarose gel using gel extraction kit. The purified TS14 amplicon as well as the pET32a(+) expression vector were double digested with *Nco* I and *Hind* III restriction enzymes following standard protocol for ligation of the product in the reading frame. *Escherichia coli* DH5α competent cells were transformed with the recombinant pET32a plasmid construct containing TS14 gene insert and the positive clones were identified by colony PCR using the same pair of primers (Sambrook *et al.* 1989). Further confirmation of the transformed colonies was made by restriction enzyme digestion of the recombinant plasmid DNA using *Nco* I and *Hind* III carried out at 37 °C for 1 h. The digested products were visualized on a UV transilluminator after electrophoresis in ethidium bromide stained 1.4% agarose gel at 3 V/cm constant current for 60 min.

A positive clone, harboring the TS14 gene sequence, was picked up for custom sequencing of nucleotides from the Department of Biochemistry, Delhi University, New Delhi. The sequence was blasted against the similar sequences present in the database and sequence alignment and analysis was performed using DNASTAR Version 5.0. Phylogenies were constructed by neighbor-joining method with p-distance model through homogeneous pattern among lineages and tested by bootstrap with 1000 replicates, using MEGA version 4.0 (Tamura *et al.* 2007).

Expression, purification and western blotting of rTS14: The thioredoxine fused recombinant TS14 protein was expressed using pET32a(+) expression vector. *E. coli* BL21 codon(+) cells were transformed with pET32a(+) plasmid construct containing TS14 gene insert and induced with 1 mM IPTG. The expressed recombinant protein was purified under denaturation condition by affinity chromatography using Ni-NTA agarose with certain modifications introduced in the purification parameter and purity of the eluted protein was checked by SDS-PAGE using 12% gel under denaturing conditions at 100 V for 2–3 h. The gel was stained with staining solution and destained for visualization of the expressed protein. The immunoreactivity of the recombinant protein was further evaluated by western blotting against infected pig sera (8)

and with hyperimmune serum raised in rabbit against the whole cyst antigen. In addition, immunoreactivity against pig sera extracted from known cysticercosis negative and hydatidosis positive cases were also evaluated using western blotting.

RESULTS AND DISCUSSION

Molecular cloning and characterization of TS14 gene:

The coding sequence of TS14 gene of *T. solium* cysticerci was PCR amplified using *de novo* primers with an annealing temperature of 56°C. The amplified product was resolved as a single band of 280 bp (Fig. 1). Colony PCR, using the specific primers, amplified a 258 bp product and the insert of specific 258 bp was released by *Nco* I and *Hind* III endonuclease digestion of the plasmids extracted from the transformed *E. coli* cells. The nucleotide sequence of TS14 was submitted to GenBank (Accession Number KM047087).

The nucleotide and amino acid sequences of TS14 gene of *T. solium* (Indian isolate) was aligned against the sequences reported from China, Peru and Mexico and analyzed by DNASTAR software. The 258 bp sequence of TS14 gene encodes the protein constituting of 85 amino acid residues with theoretical molecular weight and isoelectric point of 9.62 kDa and 9.4, respectively. The *in silico* analysis of TS14 gene of *T. solium* (Indian isolate) revealed a slightly higher AT density (57%) over the total GC density (43%) with the base composition of A:92; T:55; G:59; C:52. The TS14 coding sequence of *T. solium* cysticercus (Indian isolate) bears high level of nucleotide

sequence homology to that of other isolates reported from elsewhere. It showed 99.5% homology (Fig. 2a) with the published sequences of *T. solium* cysticerci China isolate (AF356335), 99.2% homology with other two Chinese isolates (JF906195 and DQ864645) and 98.8% homology with Peru (AF082829) and Mexico (AF257776) isolates.

The deduced amino acid sequence of TS14 protein of *T. solium* (Indian isolate) revealed 98.8% homology (Fig. 2b) with a China isolate (DQ864645) and 98.5% homology with another isolate from China (AF356335). Some other isolates from China (JF906195), Peru (AF082829) and Mexico (AF257776) showed 97.6% amino acid sequence homology with TS14 of *T. solium* Indian isolate. A single nucleotide variation at position 29 of TS14, where cytosine (C) base is replaced thymine (T), resulted in replacement of leucine (L) with proline (P) residue in the TS14 protein of *T. solium* Indian and Chinese isolates. Substitution of 2 bases was observed in TS14 sequences of *T. solium* Mexico and Peru isolates in comparison to Indian and Chinese isolates. Variation of a single nucleotide guanine (G) instead of adenine (A) at position 142 in the TS14 coding sequences of the Mexico and Peru isolates resulted in replacement of Glycine (G) residue with serine (S) in TS14 protein, while the same was not observed in the Indian and Chinese isolates.

In silico analysis of the nucleotide and amino acid sequences of TS14 and its subsequent phylogenetic analysis revealed that the Indian isolate of *T. solium* has closer relationship with the Chinese isolates than the Peru and Mexican isolates (Figs 3a, b).

The constituent polypeptide of TS14 is hydrophilic in nature and lacks a potential transmembrane region and glycosylphosphatidylinositol-anchor that proves the secretory nature of TS14 which accumulates in the cyst fluid after synthesis (Hancock *et al.* 2003). The polypeptide of TS14 contains charged and polar residues and is lysine-rich. The TS14 antigen has 1 potential N-linked glycosylation site. Further, the TS14 polypeptide contains 1 cysteine residue, which may be responsible for formation of homodimers via intra- or inter-chain disulphide bonds (Greene *et al.* 2000). The amino acid sequence of TS14 protein of the Indian isolate revealed its excess lysine content (total 14 lysine residues with 16.47% frequency) over other amino acid residues, a potential N-linked glycosylation site and one cysteine residue. The findings are in agreement with that of Greene *et al.* (2000).

Expression and immunoreactivity of rTS14: High level expression of TS14 was achieved in *E. coli* BL21(codon) plus cells at 6 h of induction with IPTG. The SDS-PAGE revealed the molecular mass of rTS14 as approximately 29 kDa (Fig. 4a), which included the histidine, Trx and S tags. The specific recombinant TS14 protein was purified to homogeneity using Ni-NTA agarose beads under denaturing conditions (Fig. 4b).

The identity of the recombinant protein was established by immunoblot assay using specific Ni-NTA HRP conjugate. Further, the immunoreactivity of the expressed

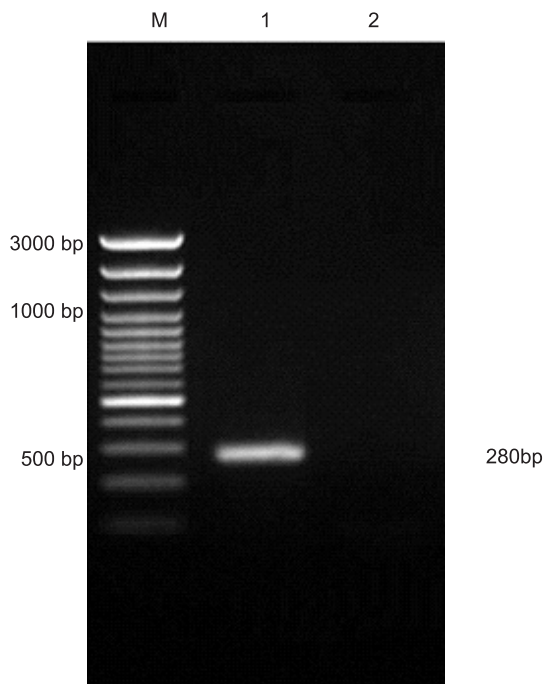


Fig. 1. Agarose (1.4%) gel showing the PCR amplification of TS14 gene fragment specific for *Taenia solium* cysticerci. Lane M, 100 bp plus DNA Marker; Lane 1, TS14 PCR product; Lane 2, negative control with water.

		Percent Identity							
		1	2	3	4	5	6		
Divergence	1	100.0	99.0	98.8	98.8	98.8	98.8	1	AF082829 Peru
	2	0.0	100.0	99.0	98.8	98.8	98.8	2	AF257776 Mexico
	3	1.0	1.0	100.0	99.5	99.5	100.0	3	AF356335 China
	4	1.2	1.2	0.5	100.0	99.2	99.2	4	Bareilly India KM047087
	5	1.2	1.2	0.5	0.8	100.0	99.2	5	China JF906195
	6	1.2	1.2	0.0	0.8	0.8	100.0	6	DQ864645 China CDS
		1	2	3	4	5	6		

Fig. 2a. Sequence pair distances of TS14 Clustal W (weighted).

		Percent Identity							
		1	2	3	4	5	6		
Divergence	1	100.0	98.5	97.6	97.6	98.8	98.8	1	AF082829 Peru
	2	0.0	100.0	98.5	97.6	97.6	98.8	2	AF257776 Mexico
	3	1.5	1.5	100.0	98.5	98.5	100.0	3	AF356335 China
	4	2.4	2.4	1.5	100.0	97.6	98.8	4	Bareilly India KM047087
	5	2.4	2.4	1.5	2.4	100.0	98.8	5	China JF906195
	6	1.2	1.2	0.0	1.2	1.2	100.0	6	DQ864645 CDS Chir
		1	2	3	4	5	6		

Fig. 2b. Deduced amino acid pair distances of TS14 Clustal W (PAM 250).

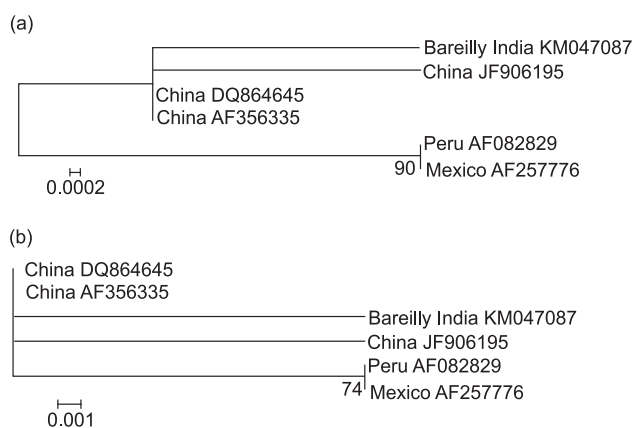


Fig. 3 (a) Phylogenetic analysis by neighbour-joining using p-distance on TS14 nucleotide sequence. Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data. (b) Phylogenetic analysis by neighbour-joining using p-distance on deduced amino acid TS14 sequence. Numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data.

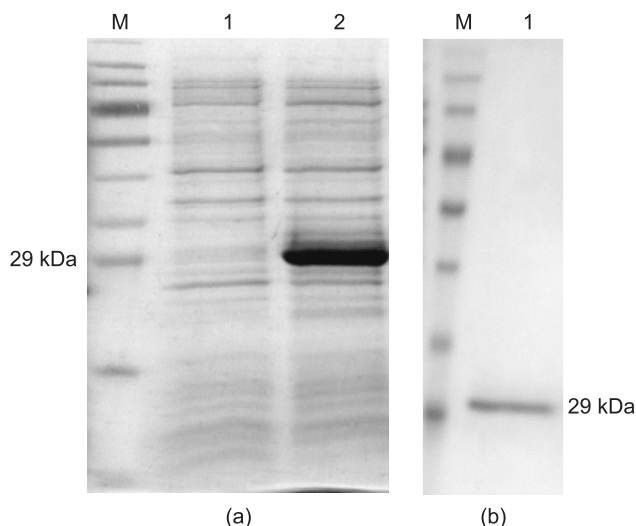


Fig. 4 (a) SDS- PAGE analysis of rTS14 expression using 12% acrylamide mix. Lane M, Molecular protein marker; lane 1, uninduced culture; lane 2, expression of TS14 fusion protein at 29 kDa. (b) SDS- PAGE analysis of purified recombinant TS14 protein using 12% acrylamide mix. Lane M, molecular protein marker; lane 1, purified rTS14 protein.

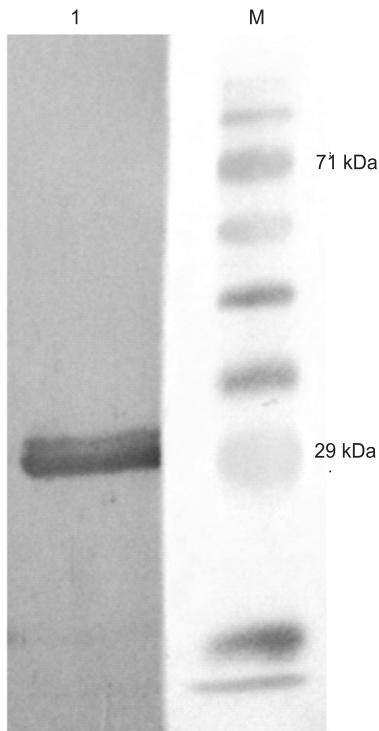


Fig. 5. Western blot showing purified recombinant TS14 reactivity to anti-sera raised in rabbit against whole cyst antigen of *Cysticercus cellulosae*. Lane M, Pre-stained protein molecular weight marker; lanes 1, reactivity of purified recombinant TS14 protein.

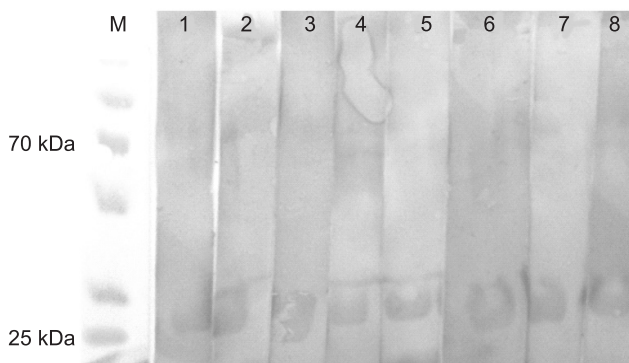


Fig. 6. Western blot analysis of rTS14 using naturally infected pig sera with *T. solium* cysticerci (Lane 1 to 8). Lane M, Pre-stained protein molecular weight marker.

protein was confirmed by western blot analysis against hyper-immune (Fig. 5) and known reference positive pig sera (Fig. 6) with strong immunoprecipitation band at 29 kDa region. Furthermore, lack of immunoreactivity against known negative sera (Lanes 1, 2; Fig. 7) and absence of cross-reactivity with sera from hydatidosis infected pig was also observed (Lane 3; Fig. 7).

Taenia solium is a gut dwelling major tapeworm of humans. Infective eggs are passed through faeces which are infective to pigs. Pigs act as the intermediate host in which the larval tapeworm, *Cysticercus cellulosae* develops in several muscles following infection acquired per os. The

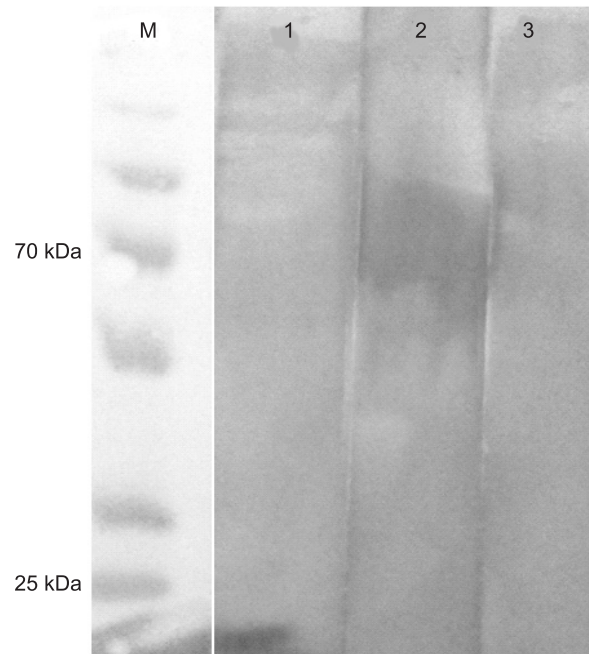


Fig. 7. Western blot showing no reactivity of purified rTS14 with negative control sera from pigs (Lane 1 and 2); with pig sera naturally infected with hydatidosis (Lane 3).

lifecycle of *T. solium* completes in human through ingestion of mealy pork. Nonetheless in certain conditions, auto-infection occurs in humans through faeco-oral/auto-infection route leading to establishment of cyst in brain. The condition is often manifested by several neurological symptoms including epileptic seizures. Diagnosis in humans is made by CT scan, though some serological tests have been reported. In the absence of suitable antemortem detection techniques for *C. cellulosae* infection in pigs, indirect serological diagnostic methods are the only available practical option. Therefore, a foolproof high throughput put tool for antemortem detection of infection in pig in a wide area is essential. TS14, a low molecular weight (9.6 kDa) secretory protein, is an important constituent of the cystic fluid of *T. solium* cysticerci, and is a promising candidate antigen for serodiagnosis of *C. cellulosae* infection in humans and pigs (Tsang *et al.* 1991, Greene *et al.* 2000, Obregon-Henao *et al.* 2003, Assana *et al.* 2007). Further, 20 of 22 amino acids in the N-terminal of TS14 are identical to the same of glycoprotein (gp) 24 and gps 39–42, which are also relevant from serodiagnostic point of view (Plancarte *et al.* 1999). This antigenic relationship between the diagnostic glycoproteins has significance as far as serodetection of this larval tapeworm infection in pig is concerned (Greene *et al.* 2000). In addition, the polyclonal antibody raised against TS14 showed reactivity with *T. solium* peptides in the Mr of 14, 18, 21, 24, and 39 to 42 kDa (Greene *et al.* 2000) which indicated that the TS14 polypeptide might be a constituent component of larger antigens ranging between 24 and 45 kDa (Plancarte *et al.* 1999, Greene *et al.* 1999). Rather, this protein shows no cross-reactivity with sera from humans infected with

Echinococcus granulosus, *E. multilocularis*, Schistosomes, *Trichinella* and *Trypanosoma cruzi* (Greene *et al.* 2000, Hancock *et al.* 2003, Scheel *et al.* 2005, da Silva *et al.* 2006), which makes it an ideal candidate antigen for serodetection of *T. solium cysticercus cellulosae*.

The TS14 protein of *T. solium* cysticerci expressed in *E. coli* under T7 promoter. A high-level expression of the protein was observed at 6 hours of induction as a prominent band of approximately 29 kDa including the histidine tag, Trx tag and S tag and the finding is consistent with previous observations of Greene *et al.* (2000). The recombinant TS14 protein containing N-terminal histidine tag contributed to its one-step purification by Ni-NTA affinity chromatography. The immunoreactivity of the expressed protein against hyper-immune sera, raised in rabbit against the whole cyst antigen, confirmed the identity of the recombinant protein.

The specific immunoreactivity of recombinant TS14 protein against serum from pigs infected with *Cysticercus cellulosae* and absence of immunoreactivity against the control pig sera in western blot format indicated the potential for its immunodiagnostic application for *T. solium* cysticercosis in pigs. The recombinant TS14 protein did not show cross reactivity with serum sample from pig infected with hydatidosis. The finding is in complete agreement with the work of da Silva *et al.* (2006). Earlier studies showed the high specificity and sensitivity of both the native and recombinant TS14 protein from *T. solium* cysticercus in ELISA and Western blot formats for detection of infection in both humans and pigs (Greene *et al.* 2000, Obregon-Henao *et al.* 2003, Assana *et al.* 2007, da Silva *et al.* 2006, 2012). The ante-mortem detection of *Cysticercus cellulosae* in pigs is currently based on tongue palpation in India. Though, the technique is highly specific, it lacks sensitivity, especially for light infections (Boa *et al.* 2002, Phiri *et al.* 2006, Soares *et al.* 2006). In contrast, the serological tests have distinct advantages over the traditional tongue palpation technique as they are more sensitive, relatively inexpensive and easy to perform on a large number of samples (Dorny *et al.* 2003).

This is the first report on molecular expression and characterization of TS14 gene of *T. solium* cysticerci for its potential serodiagnostic applications for *C. cellulosae* infection in pigs from India. However, further work on validation of the diagnostic potential of the recombinant *T. solium* TS14 protein using more number of sera samples from pigs and humans as well is required.

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