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Original Article

Optimization of In-House Indirect-ELISA & EITB Assays Employing *Cysticercus cellulosae* Antigens for Serological Detection and PCR Assays for Molecular Detection of Porcine Cysticercosis

***Kaushik Satyaprakash [1], Wiqar Ahmed Khan [1], Sandeep Prabhakarrao Chaudhari [1], Shilpshri Vasant Shinde [1], Sunil Waman Kolte [2], Nilesh Ramchandra Pansare [1], Amrut Vijay Likhite [1]**

1. *Department of Veterinary Public Health and Epidemiology, Nagpur Veterinary College, Nagpur, Maharashtra, India*
2. *Department of Veterinary Parasitology, Nagpur Veterinary College, Nagpur, Maharashtra, India*

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***Correspondence**
Email:
kaushikovc36@gmail.com

Abstract

Background: Porcine cysticercosis, caused by metacestodes of *Taenia solium* is an important neglected zoonosis. We evaluated the presence of anti-cysticercal antibodies and *T. solium* specific DNA in pig sera and blood samples respectively collected from Maharashtra, India.

Methods: A total of three antigens (Scolex Antigen (SA), Membrane Body Antigen (MBA) and Excretory-Secretory Antigen (ESA)) were prepared from metacestodes of *T. solium* and employed in an in-house developed indirect-IgG ELISA for serological screening of 1000 porcine sera samples at Department of Veterinary Public Health, Nagpur Veterinary College, Maharashtra, India. The ELISA positive sera samples were subjected to EITB Assay for detection of immunodominant peptides. An effort has been made for molecular detection of porcine cysticercosis by PCR assay targeting *large subunit rRNA* gene of *T. solium* from blood samples of the corresponding ELISA-positive pigs.

Results: The overall seroprevalence of porcine cysticercosis employing SA, MBA and ESA was 12.6%, 8.7% and 12.5% respectively. The lower and medium molecular weight peptides were the most frequently recognised in EITB assay. The numbers of bands recognised in EITB assay were observed to be proportionate with the corresponding ELISA O.D. values. An amplification product of 286 bp was observed in 22.98% (20/87), 30.35% (30/99) and 17.14% (12/70) of the sero-positive samples against SA, ESA and MBA respectively.

Conclusion: EITB still remains the gold standard serodiagnosis test for cysticercosis. The inclusion of a greater number of positive samples and purification of antigens may improve the diagnostic efficacy of the tests.

Introduction

Taenia solium is a zoonotic cestode; whose larval stage is responsible for cysticercosis, an important neglected zoonoses of great economic and public health importance in many developing countries of Asia, Africa and Latin America (1). The life cycle of the parasite includes pig and human being in which the former acts as an intermediate host and the later as definitive host. Human beings acquire cysticercosis through consumption of food and water contaminated with *T. solium* eggs excreted by tapeworm carriers (2). Neurocysticercosis (NCC), one of the most important causes of acquired epilepsy in developing countries, occurs when cysticerci develop in brain or spinal cord (3).

Skeletal muscles, liver and brain are the main predilection sites for the cysticerci in pigs, but the cysts can also be found in heart, spleen and diaphragm. Infected pigs usually remain asymptomatic (4). Tongue inspection is a highly specific ante-mortem diagnostic method for cysticercosis in pigs, but moderately sensitive for a low cyst load (5-7).

The development of an immunodiagnostic test that detects specific antibodies in either sera or cerebrospinal fluid is necessary because of its simplicity and reliability (8). Serological tests like ELISA can be applied on a large scale (9) which helps to detect light or recent infections (10). Enzyme-linked Electro-immuno Transfer Blot (EITB) Assay allows the identification of specific antigenic peptides (11). Immunodiagnosis of cysticercosis depends largely on good, potent and purified antigens *viz.* excretory-secretory, membrane and scolex extracts and somatic antigens (12-15) prepared from metacestodes of *T. solium*.

The greatest contribution of DNA-based technology has been in the genotyping of genus *Taenia* to understand the genetic diversity. PCR tests targeted at large subunit *rRNA* (*LSU rRNA*) gene of *T. solium* have been documented (16-17).

Considering this, the present study was designed to evaluate IgG-ELISA and EITB as immunodiagnostic tools using Scolex Antigen (SA), Membrane Body Antigen (MBA) and Excretory Secretory Antigen (ESA) prepared from the metacestodes of *T. solium* in order to screen the porcine sera samples collected from Nagpur and Mumbai region of Maharashtra, India. An attempt has been made for molecular detection of cysticercosis from respective blood samples by PCR test targeting *LSU rRNA* gene of *T. solium*.

Materials and Methods

Collection of Taenia solium cysts

The study was conducted in the Department of Veterinary Public Health and Epidemiology, Nagpur Veterinary College, Nagpur, Maharashtra, India. *T. solium* cysts were collected from a naturally infected pig from a local slaughter house at Nagpur India; washed in Phosphate Buffer Solution (PBS) (pH 7.2-7.4) containing 0.01% Streptomycin and 0.01% sodium azide and stored in PBS at 4 ˚C. The study was approved by the Institutional Animal Ethics Committee of Nagpur Veterinary College, Nagpur.

Collection of blood and sera samples

Blood samples of pigs slaughtered at Deonar abattoir, Mumbai (n=843) and local slaughter houses, Immamwada, Nagpur (n=157) were collected and the sera were separated at laboratory.

Molecular confirmation of cysts

Whole DNA was extracted from the cysts (one from Nagpur and two from Mumbai) using DNeasy Tissue Kit (Qiagen). The Polymerase Chain Reaction (PCR) assay targeted at *LSU rRNA* gene of *T. solium* revealed an amplified product of 286 bp upon electrophoresis, confirming the cysts to be that of *T. solium* (data not shown). The PCR products were

sequenced and submitted to NCBI (Accession No. MH084945, MH244503, MH252426).

Preparation of Antigens

Scolex antigen (SA) was prepared according to a protocol previously described (12) with slight modifications. Scolices were carefully dissected from the cysts and transferred immediately into cold PBS (pH 7.2-7.4) containing streptomycin and 5mM phenyl methyl sulphonyl fluoride (PMSF) (Sigma Aldrich, USA). These were blotted dry, homogenized followed by sonication (20 kHz, 1 mA, 60 sec) (Omni Ruptor, UK) and centrifugation (14000 rpm, 60 min, 4^0C (Remi, India). Supernatant was used as SA.

Membrane Body Antigen (MBA) was prepared from the metacestodes of *T. solium* as per the above described protocol after removing cystic fluid and scolices.

Excretory-Secretory Antigen (ESA) was prepared according to a previously described protocol (18) with some modifications. Intact cysts (n=10) with 10 ml of RPMI 1640 media (Hi-media) containing penicillin (1000 mg/L), gentamicin (2 mg/L), cefotaxime (20 mg/L) and amphotericin B (2 mg/L) were transferred into tissue culture flask (25 cc vented cap, Hi-media) and incubated at 37 ^{0}C with 1% CO_2 (Applied Biosystems). The media were harvested after 24 hours with subsequent replenishment with fresh media for seven consecutive days. The harvest was centrifuged (14000 rpm, 30 min, 4 ^{0}C) and the supernatant was saturated at 90% with ammonium sulfate. The precipitate obtained after centrifugation of saturated solution was dissolved in cold PBS (pH 7.2-7.4) and dialysed against the same buffer at 4^0C followed by concentration using Polyethylene glycol (PEG) 20000. The final volume was stored at -20^0C after addition of 1µl/ml of 5 mM PMSF. The protein concentration was estimated (19).

Indirect ELISA

The indirect ELISA was standardized by checker board analysis (20). Briefly, polyvinyl microtitre plates (Nunc, Denmark) were coated with 100 µl of 0.05 M coating buffer (pH 9.6) containing antigen in the concentration of 0.25 µg/well (for SA), 0.125 µg/well (for ESA) and 0.5 µg/well (for MBA). Sera (1:200 in PBS), Anti-pig IgG (whole molecule) HRPO conjugate (Sigma Aldrich, USA) (1:10000 in PBS) were added (100 µl/well) in subsequent steps with incubation of 2 hr and 1 hr at 37 ^{0}C respectively. The reaction was developed with O-phenylene-diamine dihydrochloride (OPD) (Sigma Aldrich, USA) and the absorbance was measured at 492 nm with ELISA reader (Multiskan Go, Thermo Fisher, Finland). A positive ELISA result was defined as two standard deviations above the mean OD value of negative sera (21).

EITB Assay

In order to assess the immunodominant proteins, EITB assay was performed on seropositive and about 40 sero-nonreactive samples (on the basis of Indirect ELISA results against all the three antigens) according to a standard protocol (22). Briefly, the antigens were subjected to Glycine Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (Glycine SDS-PAGE) (23) and transferred onto the nitrocellulose membrane (NCM) (Novex Life Technologies, Israel) using a dry transfer apparatus (i-blot, Invitrogen). The NCM was processed for development using serum (1:100), anti-species IgG HRPO conjugate (1:10000) and 3,3'- diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Aldrich, USA) at subsequent steps. Positive reaction was determined by appearance of clearly defined brown colour bands.

DNA isolation from blood samples and molecular identification of cysticercosis

Whole DNA was isolated from blood samples as per Martins et al. (2008) in a previously described protocol (24) with some modifications. Briefly, 1 ml of RBC lysis buffer (0.22% NaCl, 0.015% Saponin, 1mM EDTA; pH 7.5) was added to 400 µl of blood and centrifuged

(10000 rpm for 3 min) (Remi, India). The pellet was resuspended in 750 µl of RBC lysis buffer and the procedure was repeated to obtain a clear pellet. It was suspended in 100 µl of PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.5% Tween-20 and 100 µg proteinase K/ ml), incubated in a water bath (56^{0}C for 2 hr) and stored at -20^{0}C to be used as DNA template.

For molecular identification of cysticercosis, blood DNA was subjected to conventional PCR against *LSU rRNA* gene of *T. solium* using the primers TBR-3 (5'-GGCTTGTTTGAATGGTTTGACG-3') and TBR-6 (5'-GCTACTACACCTAAATTCTAACC-3') as forward and reverse primers respectively according to a previously described protocol (16) followed by electrophoresis on 1.5 % agarose gel.

Results

The protein concentration of SA, MBA and ESA was estimated to be 0.9725 µg/µl, 1.0903 µg/µl and 1.1271 µg/µl respectively. The cut-off O.D. values in ELISA employing SA, ESA and MBA was 0.1057, 0.1103 and 0.1081 respectively (Fig. 1).

The overall seroprevalence of cysticercosis employing SA, ESA and MBA was given in Table 1. Above all, 1.9% of samples were observed to be sero-positive against all the three antigens employed in the study.

Table 1: Seroprevalence of cysticercosis in pigs by indirect ELISA employing SA, ESA and MBA

Antigens	***Place of Collection***		***Total*** *(n= 1000)*
	Mumbai (n= 843)	**Nagpur (n= 157)**	
	Positive (%)	**Positive (%)**	**Positive (%)**
Scolex Antigen (SA)	87 (10.32)	39 (24.84)	126 (12.60)
Excretory-Secretory Antigen (ESA)	99 (11.74)	26 (16.56)	125 (12.50)
Membrane Body Antigen (MBA)	70 (8.30)	17 (10.82)	87 (8.7)

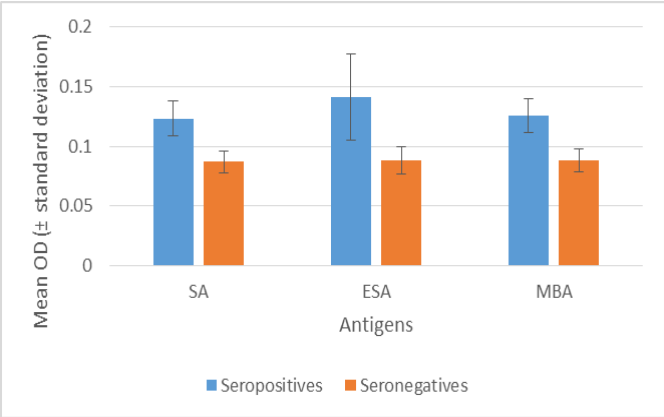

Fig. 1: Distribution of mean O.D. values in sero-positive and sero-negative pig samples by indirect ELISA employing SA, ESA and MBA

EITB assay was performed on 19 sera samples which revealed ELISA positivity for all the three antigens along with 40 sero-negative samples (ELISA negativity against all the three antigens). Evaluating the scenario for SA, the overall immunodominant proteins were ob-

served in the range of 29-44, 44-68 and 68-98 kDa which were represented by 7, 12 and 14 sera samples respectively (Fig. 2). The immunodominant proteins for ESA were observed in the range of 16-29 (in 12 samples), 44-68 (in 11 samples) and >98 kDa (in 11 samples) respectively (Fig. 3). The immunodominant bands in the range of 44-68 and 68-98 kDa were developed by 15 and 16 sera samples respectively against MBA (Fig. 4). Out of 40 sero-negative samples, EITB reaction was noted in 3, 5 and 2 samples against SA, ESA and MBA respectively revealing the band pattern in higher molecular weight range. The number of immunodominant bands recognized in EITB assay by ELISA-positive sera samples employing the respective antigens has been presented in Fig. 5.

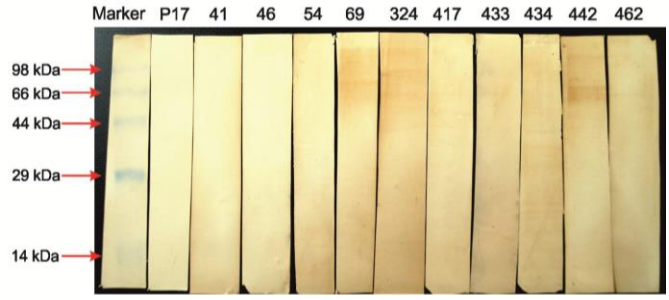

Fig. 2: EITB of sero-positive samples against SA. The numbers designate the sample numbers. The arrows indicate M_r Protein standards in kDa

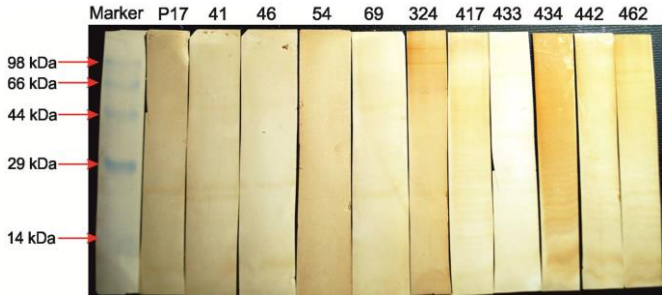

Fig. 3: EITB of sero-positive samples against ESA. The numbers designate the sample numbers. The arrows indicate M_r Protein standards in kDa

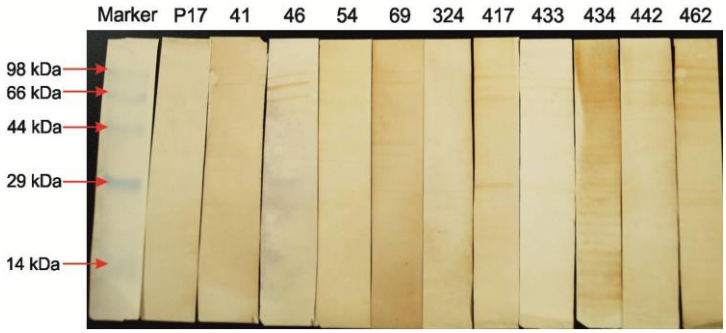

Fig. 4: EITB of sero-positive samples against MBA. The numbers designate the sample numbers. The arrows indicate M_r Protein standards in kDa

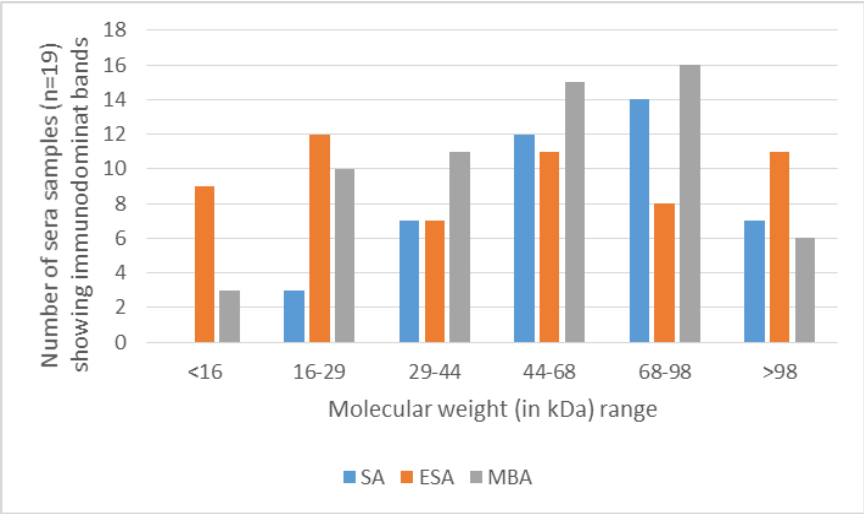

Fig. 5: The number of immunodominant bands for SA, ESA and MBA recognized in EITB assay by the ELISA positive pig sera samples (n=19)

When the ELISA O.D. values of 19 sero-positive samples were compared with the EITB pattern according to their respective antigens, the number of bands were found to be increased with proportionate higher ELISA O.D. values barring a very few samples (Fig. 6). Based on the literatures available, it seems to be the first attempt for molecular detection of porcine cysticercosis from blood samples by PCR test targeting *LSU rRNA* gene of *T. solium*. A specific amplification product (286 bp) was observed in 22.98% (20/87) of sero-positive samples against SA, 30.35% (30/99) against ESA and 17.14% (12/70) against MBA along with 6% of sero-negative samples (n=100) (Fig. 7). Amongst the samples (n=14) sero-positive against all the three antigens, only 3 were found to be PCR positive.

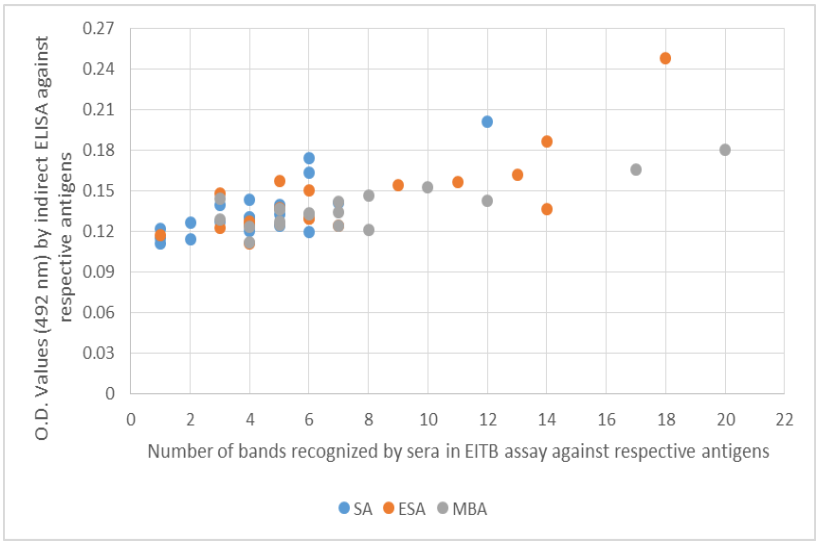

Fig. 6: Correlation between ELISA O.D. values and number of immunodominant bands recognized by EITB assay for SA, ESA and MBA

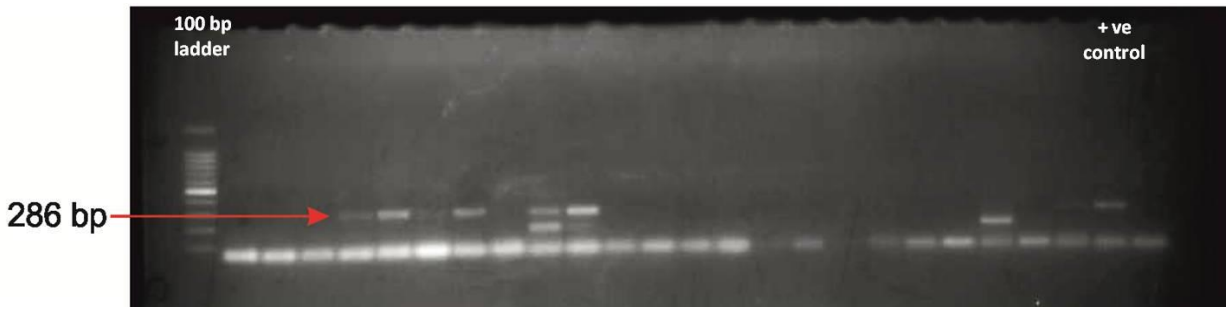

Fig. 7: PCR test targeted at *LSU rRNA* gene of *Taenia solium* for detection of cysticercosis in pigs. DNA extracted from the metacestodes of *T. solium* was used as positive control

Discussion

The overall seroprevalence of cysticercosis using SA, ESA and MBA in this study could be compared to a study in Andhra Pradesh, India where a seroprevalence of 6.22-6.50 % using SA and cyst wall antigen were reported (25). However, seroprevalence of 33% using ESA in which cysts could not be detected at meat inspection (18) were observed elsewhere. Also, 65% and 45% seropositivity using SA and membrane antigens were reported in 20 MRI confirmed affected pigs (4). In another study, *T. solium* metacestode antigens were detected by monoclonal antibodies to excretory/secretory products of *T. saginata* metacestodes among 24% and 8% the free-range pigs and slaughtered pigs respectively (26). The difference in the seroprevalence of cysticercosis in pigs as observed in Mumbai and Nagpur region can be attributed to various factors *viz*., age of animals, method of rearing (intensive, semi-intensive or free ranging), disposal methods of sewage and hygienic conditions in and around pig pens and human habitations.

A higher mean O.D. value was observed in case of ESA in our study. Increasing antibody titre over parasitism may be related to the existence of viable, metabolically active cysticerci, producing and secreting substances that stimulate the host's immune system (27). A significant increase of IgG antibody titre against *Cysticercus cellulosae* SA in sera of pigs experimentally infected with *T. solium* eggs were observed after 21 days post infection till 140 days; whereas, no correlations were noted between the number of cysticerci in muscles and ELISA O.D. values (28).

The occurrence of immunodominant peptides of SA as observed in the present investigation could be compared to a study where 26.0 and 43.0 kDa peptides were reported to be highly reactive (12). However, the findings could not be discussed much due to lack of literatures on this aspect. The use of SA in EITB assay in the diagnosis of human NCC has been documented (15, 29-30) where different protein fragments (13-200 kDa) were recognized by patients' sera.

The immunodominant peptides of ESA in the range of 16-29 and 44-68 kDa in the present study could be compared to highly reactive bands of 14.0, 20.0, 34.0 and 66.0 kDa (12), 29-97.4 kDa (31) and 65, 87 and 100 kDa (32). Higher mean OD value and seroreactivity to ESA increase the probability of viable cysts, as ESA is a metabolic product of live parasite. In an experimental infection, the number of EITB bands increased with the infective dose and duration of infection (33). In our study, a good correlation was observed between O.D. values and EITB pattern, where, barring a few cases, an increased number of

bands were observed in samples with higher O.D. values. A proportionate increase in the number of bands developed in EITB with an increase in ELISA O.D. values using ESA were observed in NCC patients (34). The sensitivity and specificity of the antigens could not be discussed due to unavailability of necropsy findings. The results of EITB using MBA could not be discussed due to lack of literatures in this aspect.

The tongue examination and post mortem meat inspection are practically not possible in rural settings where the pigs are reared under free ranging system. The possible causes of sero-positivity by ELISA and EITB may be attributable to factors *viz*., low cyst burden (which may render a negative necropsy report), transfer of maternal antibodies which remain in offspring for several months, prior anthelmintics treatment and past infections (degenerated or caseous cysts) (35-36). In addition, sero-positive pigs (showing 1-2 EITB bands) were reported as negative (no cysts) at necropsy, while a 3-EITB-band pig harboured very few degenerated and healthy cysts in the whole carcass (37). Pigs reacting to 4 or more EITB bands have higher probabilities to be infected, harbouring more cysts, than pigs with less than 4 bands (36).

Reaction to different antigenic fragments in EITB may be due to variation in host's immune response, expression of various proteins during the developmental stages of the parasite, number and persistency of the parasite, existing immune evasive mechanisms, complex and diverse core epitopes of antigens, varied levels of glycosylation and sometimes the active sites might have been cleaved off during antigen preparation leading to their non-detection (14, 38-39). The variation can be explained on the basis of laboratory animal models by experimentally infecting them with eggs of *T. solium* (40). Another reason for seropositivity without actual infection might be due to cross reactivity among closely related parasites (11, 23, 41) which may share some common antigens with that of metacestodes of *T. solium*.

As far as the molecular detection of cysticercosis in pigs from the blood samples targeting *LSU rRNA* gene of *T. solium* is concerned, this seems to be the first report in this aspect as per the available literatures. The gene is specific and conserved for taeniid cestodes. It has been targeted previously for validation of the meat inspection (17) and muscle lesions (18) for confirmation of cysticercosis. In another study, a high sensitivity with good specificity was observed for ELISA, but a high specificity with low sensitivity was observed for PCR, both being performed on sera samples of pigs for ante mortem detection of cysticercosis (42).

Unlike bacteria, virus and protozoan parasites, the development of metacestodes of *T. solium* in muscles and organs depends on initial number of eggs of *T. solium* consumed by pigs. The detection of its DNA in the blood samples is a difficulty in itself. DNA in the blood may circulate on scavenger receptor of cells (42) or may have come from damaged cyst fragments. Further studies are required to better understand the mechanism and evaluate the persistence of a tissue parasite DNA in blood.

Conclusion

The present study aimed at utilization of three different antigens prepared from the metacestodes of *T. solium* for detection of anti-*Cysticercus* antibodies by indirect-ELISA and EITB assays. Overall, a good correlation between ELISA and EITB positivity was observed. The lower and medium molecular weight proteins were found to be immunodominant. Although, a specific amplification product targeted at *LSU rRNA* gene of *T. solium* could be observed by PCR, further studies are required in this aspect. Realistically, PCR and EITB have been considered to be costly in screening large number of samples, in

that case ELISA stands to be an effective serodiagnostic method. Use of purified antigens and inclusion of cysticercosis positive pig samples may improve the efficacy of the diagnostic tests.

Acknowledgements

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Conflict of Interest

The authors declare that there is no conflict of interest.

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