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Characterization of somatic and metabolic antigens of *Cysticercus cellulosae* and determination of immunodominant proteins

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ABSTRACT

The present study shows the characterization of four somatic antigens (Whole Cyst Antigen (WCA), Cystic Fluid Antigen (CFA), Scolex Antigen (SA) and Membrane-Body Antigen (MBA)) and one metabolic antigen (Excretory-Secretory Antigen (ESA)) prepared from *Cysticercus cellulosae* originated from a naturally infected pig. Immunodominat proteins were determined using hyper immune rabbit sera. The Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) profile of the antigens revealed different numbers and patterns of protein bands in the range of 11.80 to 176.74 kDa. The electrophoretic pattern of ESA also revealed a number of closely placed indistinct bands in the range of 20-34 kDa.. The proteins in the lower molecular weight range (< 30 kDa) in the case of WCA, CFA, ESA, and the medium molecular weight range (30-60 kDa) in the case of SA and MBA were observed to be immunodominant in the Enzyme-Linked Immunoelectrotransfer Blot (EITB) assay. These immunodominant proteins could be further investigated as potential diagnostic antigens in the serodiagnosis of cysticercosis in pigs, and neurocysticercosis in human patients.

Key words: cysticercosis; pig; antigens; EITB; immunodominant proteins

Introduction

Cysticercosis is a parasitic disease of pigs caused by the larval stage of the cestode *Taenia solium*. Pigs are infected by ingestion of feed, water, sewage and vegetation contaminated with the eggs of *T. solium* expelled from the human tapeworm carriers, which may be due to open defaecation or improper disposal of night soil. It is recognized by the World Health Organization (WHO) as a "major neglected disease" (COYLE et al., 2012) and is in the group of neglected tropical diseases (NTD). The adult tapeworm develops in the intestine of human beings upon consumption of infected

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pork containing the larvae of *T. solium,* leading to taeniasis in humans. Human beings aquire cysticercosis due to the faeco-oral transmission of *T. solium* eggs from tapeworm carriers (GARCIA and DEL BRUTO, 2000). Although cysticerci can develop in any organ or muscles in the body of the intermediate host, the development of cysticerci in the central nervous system (brain or spinal cord) of human beings causes neurocysticercosis (NCC), which is the most serious form of this disease. The emergence of cysticercosis is a serious threat to economic productivity and human health in many areas of South-East Asia (RAJSHEKHAR et al., 2003; ITO et al., 2004).

The lack of specificity of the neurological symptoms in pigs and humans makes it impossible to diagnose the disease on clinical grounds alone. Thus, clinical presumptive diagnosis is usually confirmed by neuroimaging techniques, serodiagnosis and epidemiological data. The only absolute diagnostic criterion is histological demonstration of the parasite from biopsy samples of the brain or spinal cord, cysts containing protoscolex on Computed Tomography (CT) scan or Magnetic Resonance Imaging (MRI), and direct visualization of sub-retinal parasites by funduscopy (GARCIA et al., 2002). These techniques are not accessible for poor people living in an endemic area because of their very high cost. Radiography, sonography or CT scan techniques are not feasible diagnostic methods in the case of pigs for ante-mortem diagnosis of cysticercosis. In most cases, the condition is diagnosed at post-mortem inspection. Therefore, the development of immunodiagnostic tests that detect specific antibodies in the serum is an urgent need (ITO et al., 2003).

Enzyme-linked immunoelectrotransfer blot (EITB) assay, using lentil-lectin purified glycoprotein (LLGPs) of *T. solium* metacestode antigens has been the "gold standard" serodiagnostic assay for NCC, with a reported 98% sensitivity and 100% specificity (TSANG et al., 1989). Immunodiagnosis of cysticercosis depends on a good, potent and purified antigen prepared from metacestodes of *T. solium,* such as: low molecular mass antigens (ATLURI et al., 2009), excretory/secretory, somatic antigens (SAHU et al., 2009), crude soluble extract (ATLURI et al., 2009), total saline extract (OLIVEIRA et al., 2007), vesicular fluid (ARRUDA et al. 2005) and membrane and scolex extracts (ARRUDA et al., 2005). The antibody titre in infected animals is sometimes quite low, and requires a highly sensitive immunodiagnostic assay such as Enzyme-Linked Immunosorbent Assay (ELISA) and western blotting. Metacestodes of closely related cestodes share some common antigenic fractions which can cross-react and decrease the diagnostic specificity (MINOZZO et al., 2008; EL-MOGHAZY and ABDEL-RAHMAN., 2012).

The native antigens of *C. cellulosae* can be broadly classified into somatic and metabolic antigens. Somatic antigens are the molecules comprising the soma (body) of the parasite, whereas metabolic antigens are the secretory and excretory molecules of the larvae during the period of active infection. The appearance of varying anti-cysticercal antibodies in the sera of infected pigs and humans at different periods of infection may be due to qualitative and quantitative changes in somatic and excretory-secretory antigens released during various phases of parasite development.

Keeping this in mind, the present study was undertaken to characterize Whole Cyst Antigen (WCA), Cystic Fluid Antigen (CFA), Scolex Antigen (SA), Membrane-Body Antigen (MBA) and Excretory-Secretory Antigen (ESA) by SDS-PAGE, and determine the immunodominant proteins by EITB assay, using hyperimmune rabbit sera raised against these antigens.

Materials and methods

Collection of larval stages (metacestodes) of Taenia solium. The larval stages (metacestodes) of *T. solium* were collected from a heavily infected pig carcass identified upon post mortem examination at a local slaughter house in Nagpur, Maharashtra. They were washed with a cold Phosphate Buffer Solution (PBS) (pH 7.2) containing 10 mg/ml Streptomycin and 0.01% sodium azide, and preserved in the same buffer at 4-8 ˚C with the addition of 1µl/ml of 5mM phenylmethylsuphonyl fluoride (PMSF) (Sigma Aldrich, USA) until further processing.

Preparation of antigens. Metacestodes of *T. solium* were processed for preparation of four somatic (Whole Cyst Antigen (WCA), Cystic Fluid Antigen (CFA), Scolex Antigen (SA) and Membrane Body Antigen (MBA)), and one metabolic antigen (Excretory Secretory Antigen (ESA)) as follows:

Whole Cyst Antigen (WCA). The Whole Cyst Antigen (WCA) was prepared according to DHANALAKSHMI et al. (2005) with some modifications. The cysts were thoroughly washed in PBS (pH 7.2) containing 50 µg/ml streptomycin, 0.01% sodium azide and 10 µg/ml PMSF. Cysticerci (10 g) were homogenized at 4^0C in 20 ml of PBS containing 0.1 mM PMSF as a preservative. The homogenate was sonicated (Ultrasonic Sonicator, Trans-O-Sonic, Mumbai) at 20kHz, 1 mA for 60 seconds, followed by centrifugation at 14000 g for 60 minutes at 4^0C (Benchtop Cooling Centrifuge, Remi, India). The supernatant was collected and stored at -20^0C to be used as the whole cyst antigen (WCA).

Cystic Fluid Antigen (CFA). The Cystic Fluid Antigen was prepared according to ARRUDA et al. (2005) with some modifications. The cysts (10 g) were thoroughly washed in PBS (pH 7.2) containing 50 µg/ml streptomycin, 0.01% sodium azide and 10µg/ml PMSF, transferred to a sterile Petri dish, and punctured with a sterile needle. The fluid (approx 6 ml) was collected aseptically and centrifuged at 14,000 g for 30 minutes at 4^0C. The supernatant was sonicated for 60 sec, followed by the addition of 1µl/ml of 5mM PMSF, and stored at -20^0C as CFA.

Scolex Antigen (SA). The Scolex Antigen was prepared according to DHANALAKSHMI et al. (2005), with slight modifications. A total of 1086 scolices, weighing 325.8 mg, were carefully dissected from the cysts with fine forceps and a scalpel, and transferred immediately into 2 ml PBS (pH 7.2) containing streptomycin and 5mM PMSF. They were dried with blotting paper, homogenized followed by sonication at 20kHz, 1 mA for 60 seconds, and then centrifuged at 14000 g for 60 minutes at 4^0C. The supernatant was used as SA.

Membrane-Body Antigen (MBA). The Membrane-Body Antigen was prepared from *C. cellulosae* after removing the cystic fluid and scolices. The cyst wall and body components (10 g) of the larvae were transferred into 20 ml PBS containing streptomycin and 5mM PMSF. The protocol was similar to that used for WCA.

Excretory-Secretory Antigen (ESA). The Excretory-Secretory Antigen was prepared according to D'SOUZA and HAFEEZ (1999) with some modifications. Ten intact cysts were placed into a tissue culture flask (25 cm^3 vented cap, Hi-media) containing 10 ml of RPMI 1640 media (Himedia) with the addition of penicillin 1000 mg/L, gentamicin 2 mg/L, cefotaxim 20 mg/L and amphotericin B 2mg/L, and incubated at 37^0C with 1% CO_2 (CO_2 incubator, New Brunswick Galaxy® 170 S). The media was harvested after every 24 hours, with subsequent replenishment with fresh media, for seven consecutive days. The harvest was centrifuged at 14000 g for 30 minutes at 4^0C, and the supernatant was saturated at 90% with ammonium sulfate. The saturated solution was centrifuged at 10000 g for 15 minutes at 4^0C, and the precipitate was dissolved in PBS (pH 7.2) followed by dialysis against the same buffer at 4^0C. The dialysate was concentrated using Polyethylene glycol (PEG) 20000 (Himedia), and the final volume was stored at -20^0C after the addition of 1µl/ml of 5 mM PMSF.

Estimation of protein content. The protein concentration of all the five antigens was determined using the method suggested by LOWRY et al. (1951). The optical density of the standard (Bovine serum albumin fraction V) and the samples was measured at 750 nm by spectrophotometer (Multiskan Go, Thermo Fisher, Finland).

Characterization of antigens. The antigens were separated electrophoretically by Glycine Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (Glycine SDS-PAGE), a method described by LAEMMLI (1970), using 12.5% separating and 5% stacking gel in a mini gel assembly (Vetrical electrophoresis mini system, Genei, Bangalore). After the electrophoretic separation, the gels were stained with 0.1 % Coomassie brilliant blue (R-250). Finally the size of the fractionated proteins was determined by the Quantity One software of the Gel Documentation System (BioRad, USA).

Raising hyperimmune sera against the antigens. Hyperimmune sera were raised in healthy adult New Zealand White rabbits (1.5-2 years old) against the respective antigens. Emulsions (of the respective antigens) comprising 250 µg of the antigen, 500 µl of PBS (pH 7.2) and 750 µl of Fruend's Complete Adjuvant (FCA) were injected sub-cutaneously into the respective rabbits. Boosters were administered at 14 days post initial inoculation. Blood samples were collected by cardiocentesis seven days after the booster injection, and the sera were separated and stored at -20^{0}C. Sera collected before the administration of antigens were used as the negative control. All work related to the use of animals was approved by the institutional animal ethics committee.

Determination of immunodominant proteins by Enzyme-Linked Immunoelectro Transfer Blot (EITB) Assay. In order to determine the immunodominant proteins of antigens recognized by the sera samples of patients, EITB assay was performed according to TOWBIN et al. (1979). Briefly, the antigens were subjected to Glycine Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (Glycine SDS-PAGE), and transferred onto a nitrocellulose membrane (NCM) (Novex Life Technologies, Israel) using dry transfer apparatus (iBlot Dry Blot System, Invitrogen), according to the manufacturer's protocol. The NCM was blocked with 5% skimmed milk in PBS (2 hours at 37^{0}C) followed by washing with PBS-Tween-20 (PBS-T). It was subsequently incubated with hyper immune rabbit serum (1:100 in PBS) at 37^{0}C for two hours, then Anti-rabbit IgG Horse Radish Peroxidase (HRPO) conjugates (Sigma Aldrich, USA) (1:10000 in PBS) at 37^{0}C for one hour, with subsequent washing with PBS-T after each step. The NCM was developed with a substrate solution containing 1 mg/ml of 3, 3'- diaminobezidine tetrahydrochloride (Sigma Aldrich, USA) and 30% hydrogen peroxide (100µl) in PBS (100ml). A positive reaction was determined by the appearance of clearly defined brown colour bands as immunoreactive proteins, and this was compared with the standard molecular weight marker using the Quantity One software of the Gel Documentation System (BioRad, USA).

Results

The protein concentrations of WCA, CFA, SA, MBA and ESA were estimated to be 1.0175 µg/µl, 1.2622 µg/µl, 0.9725 µg/µl, 1.0903 µg/µl and 1.1271 µg/µl, respectively.

The SDS-PAGE profile of the antigens revealed a varied number (25 bands for WCA, 17 for CFA, 29 for SA, 26 for MBA and 16 for ESA) and pattern (11.80 to 171.35 kDa for WCA, 12.50-176.74 kDa for CFA, 13.13 to 150.60 kDa for SA, 15.23 to 174.12 kDa for MBA and 12.00 to 164.04 kDa for ESA) of protein bands (Fig. 1).

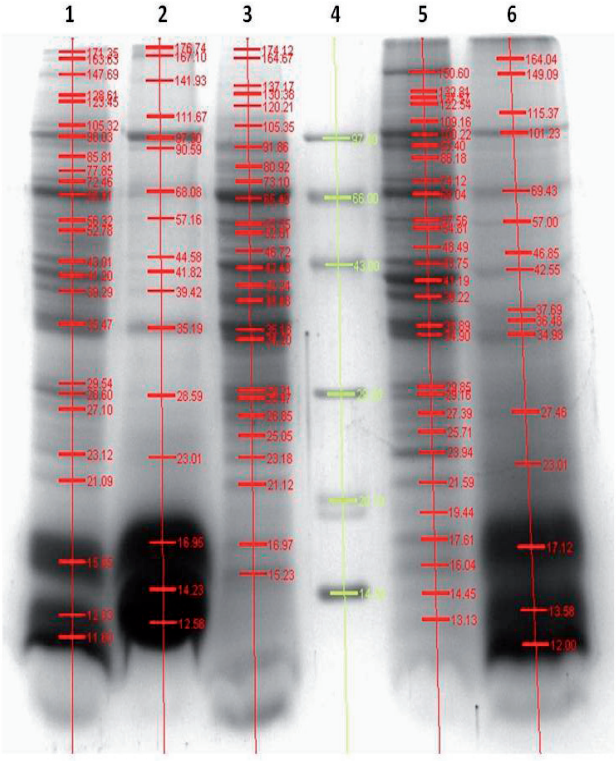

Fig. 1. SDS-PAGE profile of *Taenia solium* metacestodes antigens (Lane 1: WCA, Lane 2: CFA, Lane 3: MBA, Lane 4: Protein molecular weight marker of medium range (Genei, Bangalore), lane 5: SA, Lane 6: ESA)

The EITB assay revealed the immunodominant proteins of the antigens recognized by the respective hyperimmune sera. Against WCA, three proteins of M_r < 16 kDa (10.40 kDa, 11.52 kDa

and 14.86 kDa) were dark stained and recognized as immunodominant proteins (Fig. 2). Some of the finer bands in the high molecular weight range (>98 kDa) showed immunoreactivity with hyperimmune serum. In the case of CFA, the proteins of the lower molecular weight range (< 30 kDa) were found to be more immunogenic (Fig. 3). For SA, the bands around 35, 39, 53, 64, 76, 91 and 114 kDa were observed to be prominent on the basis of intensity (Fig. 4). Protein bands of 25, 35, 51, 61, 73 and 85 kDa were found to be immunodominant against MBA (Fig. 5). The ESA showed 10 reactive antigenic components, of which the reactive bands 12, 16, 35, 97, 121 and 129 kDa were found to be prominent (Fig. 6). With the negative control sera, no reactive bands were obtained against any of the antigens.

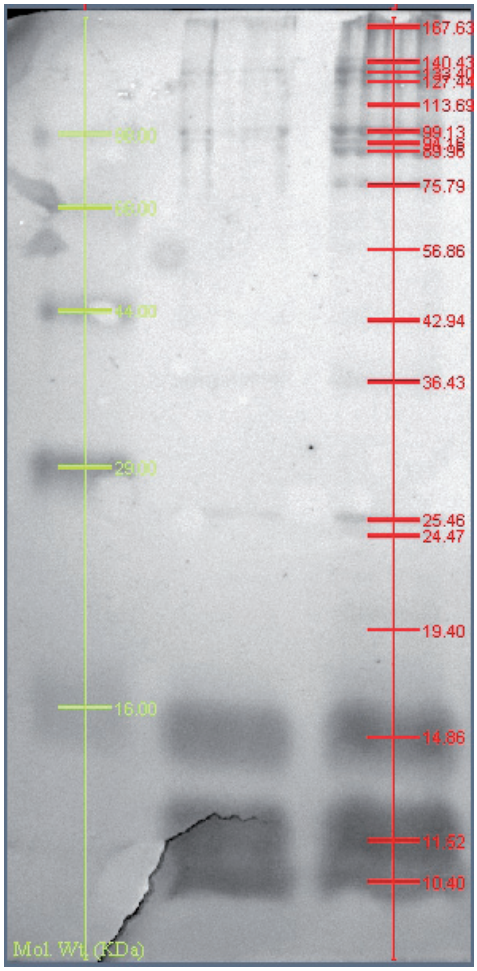

Fig. 2. EITB profile of hyper immune serum against WCA (Lane 1: Pre-stained protein marker, Lane 2 and 3: WCA)

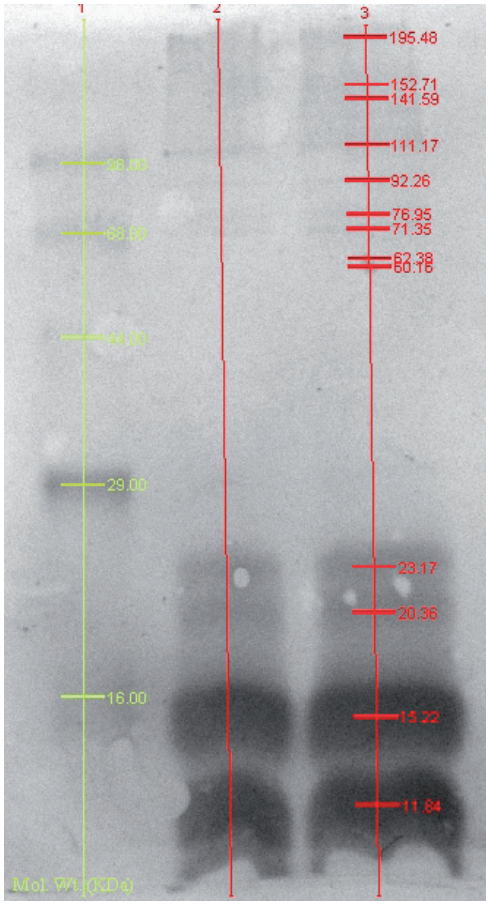

Fig. 3. EITB profile of hyper immune serum against CFA (Lane 1: Pre-stained protein marker, Lane 2 and 3: CFA)

Discussion

In this study, the SDS-PAGE profiles of the crude antigens prepared from the metacestodes of *T.solium* were found to differ from each other in terms of the number and size of the bands. Similar results were obtained by LI et al. (1998), who observed 20, 20, 21, 21 and 13 bands in the range of 6.6 to >116 kDa in whole supernatant, cyst fluid supernatant, scolices, whole cysts and urea-soluble scolex antigens after silver staining. In another study, crude antigenic components exhibited 31 protein bands in the range of 22-260 kDa (KIM and YANG, 1988) and CFA revealed 13 bands in the range of 220 to 14 kDa (JAWALAGATTI et al., 2016) compared to the 17 bands for CFA observed

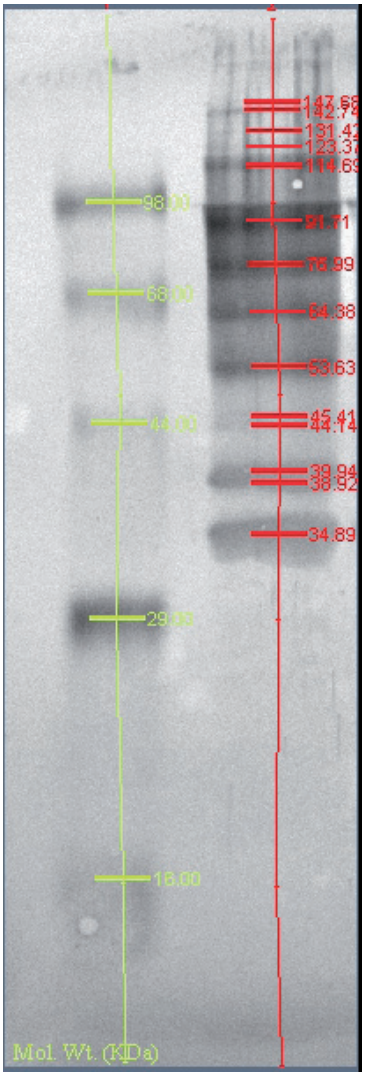

Fig. 4. EITB profile of hyper immune serum against SA (Lane 1: Pre-stained protein marker, Lane 2: SA)

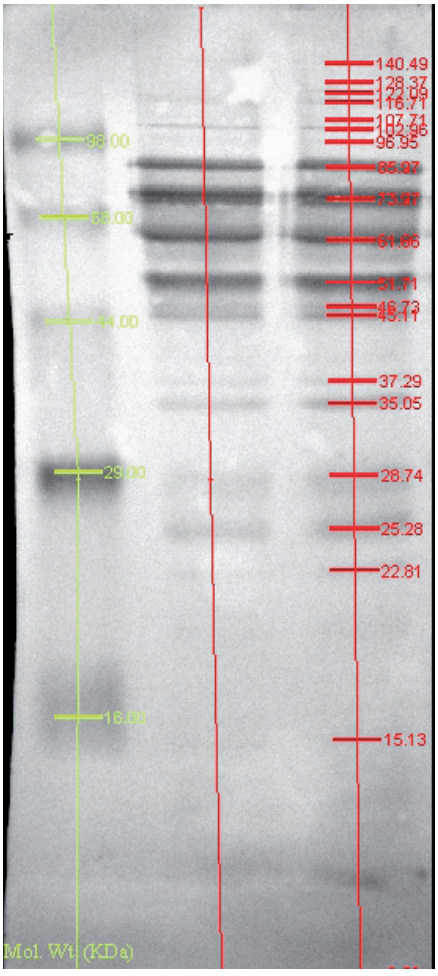

Fig. 5. EITB profile of hyper immune serum against MBA (Lane 1: Pre-stained protein marker, Lane 2 and 3: MBA)

in our study. WCA exhibited 25 bands (11.80-171.35 kDa) in contrast to 18 bands (13 kDa to >97.4 kDa) observed by DHANALAKSHMI et al. (2005). The lower molecular weight fractions of WCA and CFA were found to be prominent in this study. ITO et al. (1998) also reported three major bands in the range 10-26 kDa from intact cysts and cyst fluids; and SAKO et al. (2013) observed major bands between 10-25 kDa in low-molecular-weight antigens (LMWAgs) obtained by cation-exchange chromatography from cyst fluids. The electrophoretic pattern of SA obtained in this study coincides with the findings of CHO et al. (1987), DHANALAKSHMI et al. (2005), NETO et al. (2007) and SHUKLA et al. (2008), who reported fragments in the range of 7-200 kDa. Our results differ from the findings of BHADRIGE et al. (2014) who analyzed WCA and SA, showing 15 and 7 protein fractions in the range of 25.1- 64.9 kDa and 26.6-66.8 kDa, respectively. The ESA revealed 16 protein bands in the range of 12.00-164.04 kDa as against 12 to 29 bands (19.2-324.3 kDa) reported in other observations (KO and NG, 1998; ATLURI et al., 2009; SAHU and PARIJA, 2010). The difference might be caused by post-translational modification of the proteins.

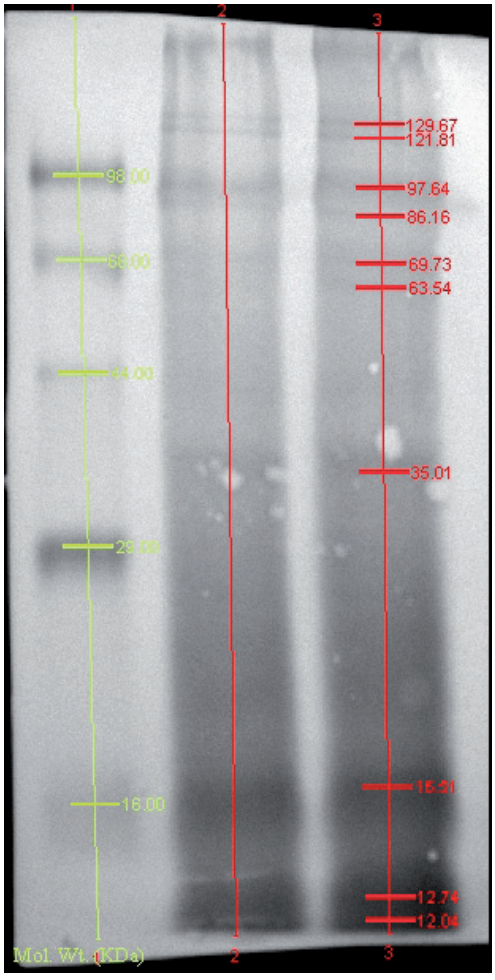

Fig. 6. EITB profile of hyper immune serum against ESA (Lane 1: Pre-stained protein marker, Lane 2 and 3: ESA)

All the antigens revealed a complex band pattern. The CFA and ESA shared at least six common bands in the range of 12-70 kDa, similar to KO and NG (1998). JIANG et al. (1987) demonstrated the highly complex nature of heat-resistant cyst fluid and scolex-cyst wall complex antigens, where the former had 16 and the latter 22 bands. Both shared three common components. SHUKLA et al. (2008) demonstrated prominent bands between 60-70 kDa and 40-45 kDa to be common to both membrane and scolex antigens. In our study, the lower molecular weight fractions of WCA, CFA and ESA exhibited a similar intensely stained pattern, but this was not observed in the case SA or MBA, suggesting these to be the immunogenic proteins of CFA only. As WCA is prepared from the whole cyst, it is supposed to contain the antigenic fractions of CFA, SA and MBA. Therefore, we obtained some similar band patterns in these antigens.

Cystic fluid, the main factor responsible for antigenic stimulation, is the secretion from the parasite during its growth in the animal's body (SIRACUSANO et al., 2012). During the *in vitro* culture of the larvae, the metacestodes secrete and excrete their metabolites into the culture medium which constitute the ESA. Some of the metabolites, excreted/secreted during the *in vitro* cultivation, might be similar to the metabolites in the cystic fluid of the larvae during natural infections. This might have resulted in the similar antigenic profile of CFA and ESA. There were some differences between the number and pattern of bands obtained in the present study and those found in the literature. The method of antigen preparation, the developmental stage of the metacestodes in the pigs, genotypes/ strain differences of the parasite, geographical distribution,and post-translational modifications (GOSWAMI et al., 2013) may attribute to the antigenic diversity.

The WCA and CFA were found to contain similar protein bands in the lower molecular weight range, as observed in the EITB pattern of the antigens using their corresponding hyper immune sera. The lower molecular weight bands (12.04 kDa, 12.74 kDa and 16.51 kDa) of ESA can be compared with the electrophoretic pattern obtained by SDS-PAGE analysis. Our ESA findings are similar to DHANALAKSHMI et al. (2005) who observed the bands of 14.0, 20.0, 34.0 and 66.0 kDa to be highly reactive when developed against pig positive serum. SAHU et PARIJA (2010) demonstrated three ES proteins, viz., 67 kDa, 43 kDa and 32 kDa, of which the 43 kDa ES protein was found to be reactive with CSF and serum samples from confirmed NCC patients. As far as the scolex antigen (SA) is concerned, 100, 95 or 26 kDa proteins (NETO et al., 2007), 13, 17, and 26 kDa proteins (LISIANE et al., 1999) gave confirmatory diagnosis in neurocysticercosis positive cases. These protein bands were also observed in our study. Immunoreactive bands between 60-70 kDa

and 40-45 kDa were observed by SHUKLA et al. (2008) in the membrane and scolex extracts of *Cysticercus cellulosae* and *Cysticercus fasciolaris* for immunodiagnosis of neurocysticercosis. With the negative control sera, no reactive bands were obtained against any of the antigens, indicating that all of the immunoreactive proteins were developed due to the administration of antigens, and none of the antigenic fragments have epitopes for host antibodies.

Some of the proteins in the higher and medium molecular weight range were found to be a false positive reaction and may cross react with other parasite antigens, as already described in the literature, such as vesicular fluids of *Echinococcus granulosus* (KONG et al., 1989), crude antigen extracts of *Echinococcus granulosus, Fasciolopsis buski,* larval *Taenia hydatigena, Hymenolepis diminuta* and *Dipylidium caninum* (CHENG and KO, 1991), *Taenia crassiceps* vesicular fluid (KUNZ et al., 1989; EL-MOGHAZY and ABDEL-RAHMAN, 2012), against the sera from pigs or human neurocysticercosis patients. Some authors have also described the use of heterologous antigens obtained from *T. crassiceps* (MINOZZO et al., 2008) and *Cysticercus longicolis* (VAZ et al., 1997; BUENO et al., 2000; PARDINI et al., 2002; PERALTA et al., 2002; ISHIDA et al., 2006) in the serodiagnosis of human neurocysticercosis, as these parasites share some common antigenic fractions prepared from crude whole cyst extracts or vesicular fluids, which could be used for diagnostic purposes.

The pattern of the antigen bands obtained by SDS-PAGE analysis and that obtained after EITB, were similar. There was a minor variation in the size of the bands obtained after SDS-PAGE when compared to their EITB counterparts. This may be due to the difference in the relative migration of protein in the gel, which may vary according to the conditions and experiments, and the identification of bands and their calculation by the software.

The number of antigenic bands decreased when the antigens were tested with pig positive serum, which may be due to the fact that the non-specific epitopes, which are reactive with rabbit antiserum, are not reactive with the pig positive serum. It might also be due to the developmental stage of the cysts during natural infections, which accounts for the different expression of proteins.

Conclusion

In the present study, the hyperimmune sera against the antigens revealed the lower molecular weight protein fractions to be immunodominant in WCA, CFA and ESA, while the medium molecular protein fractions were found to be more immunoreactive in the case of SA and ESA. These immunoreactive proteins should be further researched from a diagnostic point of view, and for detection of cysticercosis in pigs and neurocysticercosis in human epileptic patients. These protein fractions may be purified from the crude antigens to increase their diagnostic sensitivity and specificity, and to rule out the occurrence of cross reactivity and false positivity.

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Conflicts of interest
There are no conflicts of interest.

Ethical standards
The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guidelines on the care and use of laboratory animals.

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SATYAPRAKASH, K., W. A. KHAN, N. N. ZADE, S. P. CHAUDHARI, S. V. SHINDE: Somatski i metabolički antigeni te imunodominantni proteini larvi *Cysticercus cellulosae*. Vet. arhiv 92, 399-409, 2022.

SAŽETAK

U istraživanju je provedena karakterizacija četiriju somatskih antigena (*Whole Cyst Antigen* - WCA, *Cystic Fluid Antigen* - CFA, *Scolex Antigen* - SA i *Membrane Body* – MBA), te jednog metaboličkog antigena (ekskrecijsko sekrecijski antigen - ESA), pripravljenih od larvi *Cysticercus cellulosae*, koje potječu od prirodno zaražene svinje. Imunodominantni proteini određivani su upotrebom hiperimunosnog seruma kunića. Profil antigena, određen elektroforezom u natrijevu dodecilsulfatnom poliakrilamidnom gelu (SDS-PAGE), pokazao je različite brojeve i uzorke proteinskih traka u rasponu od 11,80 do 176,74 kDa. Eleketroforetski uzorak ESA također je pokazao niz usko smještenih i nejasnih traka u rasponu od 20 do 34 kDa. Testom EITB (*Enzyme-Linked Immunoelectrotransfer Blot*), u slučaju WCA-a, CFA-a, ESA-e ustanovljeno je da su imunodominantni proteini manje molekularne mase (< 30 kDa), a u slučaju SA-a i MBA-a imunodominantni su proteini bili srednje molekularne mase (30 – 60 kDa). Ovi se imunodominantni proteini mogu dalje istraživati kao potencijalni dijagnostički antigeni u serološkoj dijagnostici cisticerkoze u svinja i neurocisticerkoze u ljudi.

Ključne riječi: cisticerkoza; svinja; antigeni; EITB; imunodominantni proteini