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Evaluation of excretory/secretory products of larval *Taenia solium* as diagnostic antigens for porcine and human cysticercosis

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Abstract

Excretory/secretory antigens (ES) of larval *Taenia solium* were obtained by maintaining the bladder worms in Medium 199 for 3 days. Analsysis by SDS-PAGE showed that ES antigens consisted of at least 19 polypeptides, with M_r ranging from 14–116 kDa. Analytical isoelectric focusing revealed eight bands with acidic pI. An immunocytolocalization study using the peroxidase method demonstrated the presence of ES epitopes on the tegument of the wall of the spiral canals of bladder worms. The specificity of ES antigens was evaluated by EITB, ELISA and FAST-ELISA using antisera against the common parasites of Chinese pigs and man. ES antigens cross-reacted with the antiserum against larval *T. hydatigena* of pigs. However, these antigens were generally more specific in diagnosing human cysticercosis. Three host-like molecules with molecular masses 43, 58 and 66 kDa were present in the ES products.

Introduction

Serodiagnosis of cysticercosis remains difficult because of the unavailability of specific and sensitive antigens. The larva of *Taenia solium* contains too many conserved somatic antigens in both cystic fluid and wall which would cross-react with heterologous antibodies against other taeniids, hydatid cysts or even nematodes and schistosomes (Pammenter *et al.*, 1992). Seven specific bands of glycoprotein antigens have been recognized in the immunoblotting of whole cyst extracts and this assay system has been tested as a method of laboratory diagnosis (Tsang *et al.*, 1989; Armando *et al.*, 1990; Diaz *et al.*, 1992). According to Tsang *et al.* (1989), recognition of one or all seven bands should be considered positive. Plancarte *et al.* (1994) attempted to isolate the 24 kDa glycoprotein band by electroelution and suggested that dot blotting may facilitate the diagnosis of human neurocysticercosis. However, the somatic glycoprotein antigens have not been purified or mass produced.

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Excretory/secretory (ES) products of many helminths are well known to contain highly specific antigens which can be used for serodiagnosis. For example, ES products of the nematode, *Trichinella spiralis*, contain three immunodominant antigens which have been employed in commercial kits (Gamble, 1985; Ko & Yeung, 1991; Ellis *et al.*, 1994; Reason *et al.*, 1994). However, little is known about the specificity of the ES products of larval *T. solium*. Therefore, the feasibility of using ES antigens in diagnosing cysticercosis should be further explored.

The present paper reports an attempt to obtain ES antigens from larval *T. solium*, to determine the immunodominant epitopes and to evaluate their specificity in diagnosing porcine and human infections. The distribution of ES epitopes in the cystic structure is also studied.

Materials and methods

Production of ES materials

Cysts were obtained from pigs within 6 h of slaughter at the Kennedy Town abattoir, Hong Kong. After removal from the skeletal muscles, cysts were washed for 30 min in three changes of sterilized saline with 500 UI ml^{-1}

penicillin G and 500 μg ml^{-1} streptomycin. Thirty cysts were transferred (under axenic conditions in a laminar flow hood) to a 250 ml canted-neck culture flask (Costar), containing Medium 199 (Difco), 7000 UI of penicillin G, 7 mg of streptomycin sulphate and 18.2 mg of L-cysteine (Sigma). The flasks were maintained for 3 days in a carbon dioxide incubator (IR Autoflow, Nuaire) at 37°C. Cysts were examined daily under an inverted microscope to ensure that they remained in good condition. The medium was changed daily. Media from the 3 day collection were pooled and then centrifuged at 3000 × *g* in a refrigerated centrifuge (GPR, Beckman). The filtrate was concentrated firstly by ultrafiltration, using a 5K molecular cut-off membrane (Spectrum), and then by passing through Centricon 30 (Amicon). After determining the protein concentration by a protein assay kit (Biorad), the sample was stored at –20°C until use.

Somatic antigens and antisera preparation

Crude whole cyst (W) antigens were prepared by homogenizing 1 g of cysts in phosphate buffer (using a Telfon homogenizer), following the method of Cheng & Ko (1991). After overnight extraction at 4°C, the homogenate was centrifuged for 30 min at 3000 × *g* in a refrigerated centrifuge (GPR, Beckman). The supernatant was then centrifuged for 60 min at 25,000 × *g* and 4°C in a refrigerated ultracentrifuge (LSM, Beckman). After centrifugation, the supernatant was desalted by passing through a column packed with P6DG gel (Biorad).

Fluid (F) antigens were prepared by aspiring the cystic fluid with a 27 gauge needle. It was then centrifuged for 30 min at 15,000 × *g* and 4°C in a refrigerated ultracentrifuge (LSM, Beckman).

Crude worm extracts were also prepared from the following common parasites of Chinese pigs: larval *Taenia hydatigena, Taenia* sp. from Taiwan, larval *Echinococcus granulosus, Fasciolopsis buski, Trichinella spiralis, Gnathostoma hispidum, Trichuris suis, Stephanurus dentatus, Metastrongylus apri, Ascaris suum.*

Hyperimmune antisera against *T. solium* were prepared by injecting locally bred pigs (1 month old, male) or rabbits (New Zealand white, 16 weeks old, male) with ES, cystic fluid (F), whole cyst (W) antigens of larval *T. solium*. Small quantities, i.e. 0.5 mg ml^{-1} (for rabbits) or 6 mg ml^{-1} (for pigs) of antigen in Freund's complete and incomplete adjuvants were used for three injections at weekly intervals (Cheng & Ko, 1991). The animals were anaesthetized with ether or sodium pentobarbital (Nembutal, Abbotts Lab) and blood was collected from the marginal veins of the ear or by cardiac puncture.

Hyperimmune sera against the common parasites of Chinese pigs and pig muscles were produced in rabbits. Normal rabbit and pig sera served as negative controls.

Infected human sera were collected from 39 patients admitted to various hospitals in Hong Kong who were suspected to be suffering from cysticercosis due to the following symptoms: meningitis, epileptic fits, presence of cystic lesions in brain or other tissues. Heterologous antisera were collected from patients with other parasitic diseases. Normal control sera were collected from staff members of our laboratory.

SDS-PAGE and EITB

For the analysis of antigenic epitopes of ES products, the methods of Ko & Yeung (1989) were followed. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) in a Mini-Protean II cell (Biorad). The gels were stained either with Coomassie Brilliant Blue R250 or a silver staining kit (Sigma). Molecular mass was determined using Dalton Mark VIII markers (Sigma).

Enzyme-linked immunotransfer blotting (EITB) was performed on nitrocellulose paper (0.45 μm, Schleicher & Schnell) in a Mini-Transblot cell (Biorad). The free-binding sites were saturated with 3% gelatin. The anti-swine and anti-rabbit IgG conjugated with horse-radish peroxidase (HRPO) (heavy and light chain specific, Cappel) were diluted 1:500 and 1:2000 (v/v) respectively with 4-chloro-1-napthol being used as the substrate.

Isoelectric focusing

Analytical isoelectric focusing (AIEF) was carried out in an automatic electrophoresis system (Phast System, Pharmacia) at 2000 volts. Precast phastgels with pI 3–9 and 4–6.5 were used. The gels were stained with a silver staining kit (Pharmacia).

Enzyme-linked immunosorbent assays

To evaluate the specificity of ES antigens in diagnosing porcine cysticercosis, the Falcon assay screening test (FAST) was compared with the classical double-antibody enzyme-linked immunosorbent assay (ELISA) in an attempt to find a suitable assay for use in abattoirs. Due to a limited supply of ES antigens, only the double-antibody ELISA was used to test the human sera.

For each assay, the optimal concentrations of antigen, antibodies, and enzyme conjugate were determined by chequer-board titrations. The absorbance (OD) for FAST and double-antibody ELISA was read with an automatic ELISA reader (MR 710, Dynatech).

The double-antibody ELISA was performed according to Ko & Yeung (1989). 1 μg of W or F antigens and 5 μg of ES antigens were used to coat polystyrene microtitration plates (Linbro, Flow Lab). The test serum was diluted 1: 100 for pig samples and 1: 200 for human samples. The goat anti-swine or human IgG-HRPO conjugate was diluted 1: 8000. Ortho-phenylene diamine (OPD) served as the substrate. Absorbance values (OD) which were greater than 3× the mean OD of the normal pool were considered as positive.

FAST-ELISA was performed according to Hancock & Tsang (1986), with modifications. The kit was purchased from Becton Dickinson and 10 μg ml^{-1} of W, 5 μg ml^{-1} of F and 1 μg ml^{-1} of ES antigens were used to coat the plastic beads. The following dilutions of serum sample and goat anti-swine IgG-HRPO conjugate (Cappel) were used: 1:100; 1:15,000 and tetramethylbenzidine (TMB) (Sigma) served as the substrate. The cut-off value was set at the mean OD of the negative control plus 3 standard deviations. The beads were first incubated with antigens in coating buffer (0.1 M carbonate-bicarbonate buffer at pH 9.6) at 4°C overnight. They were then washed in washing buffer (Tween 20-Tris buffer saline), using a

garden-spray container with a nozzle. The beads were sprayed for 20 s from a distance of 10–15 cm. After washing, the beads were lowered into the wells of the microtitration plate containing the sera. The assembly was shaken gently for 5 min on a rotary shaker. The beads were washed before adding the HRPO conjugate. After incubation for 5 min, they were washed again before adding TMB. After 5 min, the beads were removed and the absorbance of samples was read at 600 nm (test filter) and 490 nm (reference filter).

Immunoperoxidase labelling

Cysts were fixed in Bouin's solution, embedded in paraffin wax and cut at 6 μm. The sections were dewaxed in xylene and rehydrated in a decreasing series of ethanol. They were incubated in PBS before the application of 1 ml of 0.3% hydrogen peroxide to block the endogenous peroxidase activity. After 30 min, the sections were rinsed three times with PBS. Non-specific binding sites were blocked by 0.2 ml of 1:30 dilution of normal goat serum. After washing, 0.2 ml of rabbit anti-ES serum (dilution 1:64) was added. After incubation for 2 h at room temperatures, the sections were rinsed three times in PBS before the addition of goat anti-rabbit IgG-HRPO conjugate (Cappel, diluted 1:320). Diaminobenzidine tetrahydrochloride (DAB) (Sigma) (0.05%, with 0.03% hydrogen peroxide) served as the substrate. The sections (dehydrated in ethanol and xylene) were mounted in Canada balsam.

Results

Protein profile and epitope distribution

The SDS-PAGE profile of the ES products showed the presence of at least 19 polypeptides (fig. 1). This number was substantially smaller than those found in either the whole cyst or membrane extracts. Three of the bands with M_r 19, 54 and 62 kDa were heavily stained, the last one being the most prominent. However, as compared with the cystic fluid, ES products contained more bands, especially those between 20–66 kDa. They shared five common bands, i.e. 20, 48.5, 54, 66, 105.

AIEF of ES products in a pI 3–9 gel showed eight bands which were evenly distributed between pI 3.5–8.0. The most prominent bands were at pI 5.0 and 5.3.

Active ES epitopes were located only in a confined region of the larval cestode. Using the peroxidase labelling method and rabbit anti-ES serum as the developing serum, moderate positive reactions were observed along the tegument of the spiral canal of the cyst (fig. 2). Other tissues of the cyst yielded a negative reaction.

EITB

Using rabbit antiserum as the developing serum, ES antigens showed cross-reactions with the following antisera: against whole cyst extract of *T. solium*, whole cyst extract of *T. hydatigena*, normal pig serum and pig muscles (fig. 3). The most interesting observation was the presence of three cross-reactive bands, with 48, 58 and 66 kDa, in the blots against normal pig serum or muscles.

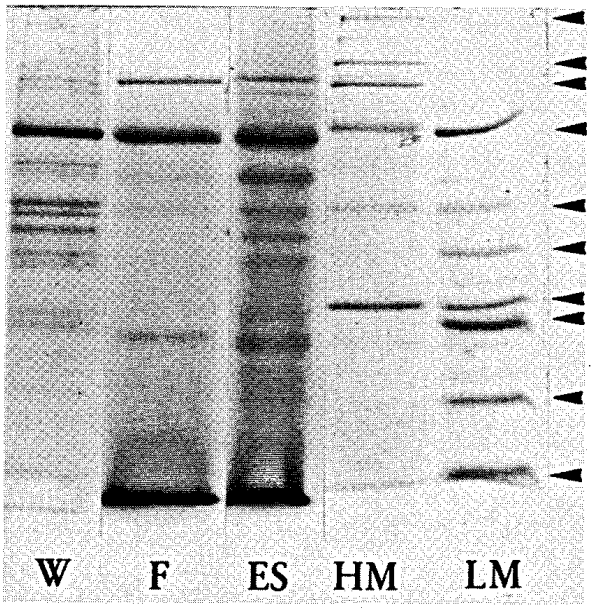

Fig. 1. SDS-PAGE profile of crude whole cyst antigens (W), cystic fluid (F) and excretory/secretory (ES) antigens of larval *Taenia solium* using 10% homogeneous gel. SDS-PAGE markers are SDS-7 (LM) and SDS-6H (HM). The molecular masses for SDS-7 are 14.2, 20.1, 29, 36, 45, 66 kDa and SDS-6H are: 29, 45, 66, 97.4, 116, 205 kDa (from bottom to top).

The 48 and 66 kDa bands were also present in the blots against *T. hydatigena* and whole cyst extract of *T. solium*. Negative reactions were observed against *A. suum*, *F. buski* and other parasites.

Using pig antiserum as the developing serum, ES antigens cross-reacted strongly with the following antiserum: whole cyst and cystic fluid of *T. solium;* cystic fluid of *T. hydatigena* (fig. 4). Fifteen active epitopes with M_r ranging from 29–97.4 kDa were detected. ES antigens reacted weakly with the following antisera: *T. spiralis*, *T. suis*, *G. hispidum*, *M. apri* and *F. buski*. Three epitopes of 40, 58 and 97 kDa were detected. Normal pig serum also reacted positively with the 58 kDa polypeptide.

ELISA

Animal sera

Using double ELISA and heterologous antisera raised in pigs as developing sera, extensive cross-reactions between ES antigens and anti-*T. hydatigena* were noted. A positive reaction with anti-*M. apri* was also observed (fig. 5). When heterologous antisera raised in rabbits were tested, a positive reaction between ES antigens and anti-pig muscles serum was observed.

In FAST-ELISA, the OD readings and OD^{+ve}/OD^{-ve} ratios of the heterologous antisera, when tested against ES antigens, were substantially lower than those of the double ELISA. But the OD readings of the positive control antisera, i.e. against whole cyst or fluid antigens of *T. solium* (fig. 6) were also lower. A positive reaction between ES antigens and anti-*T. hydatigena* serum also occurred.

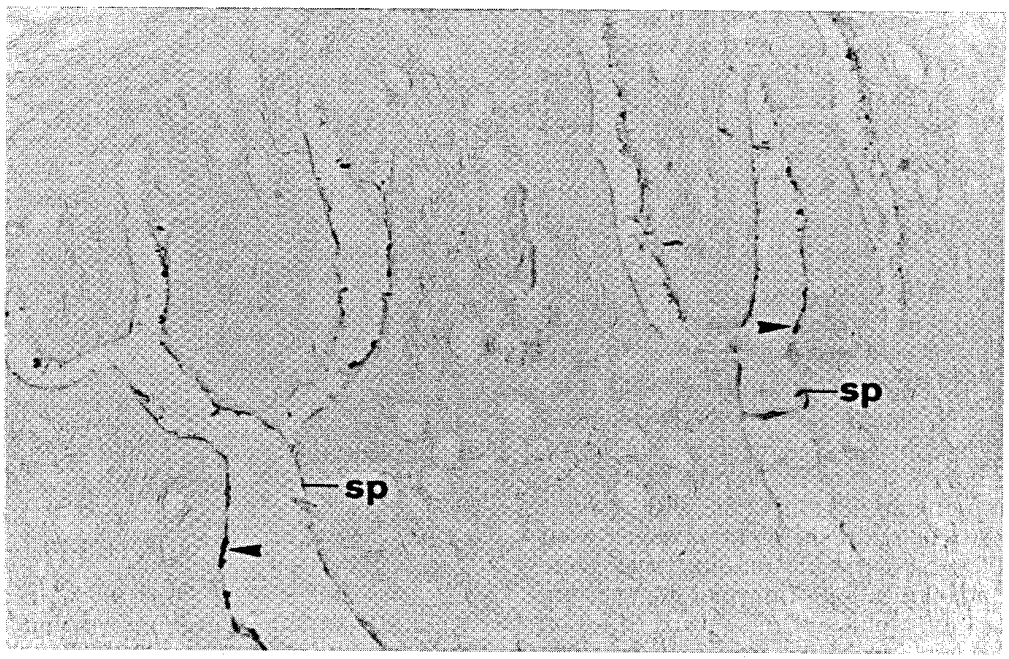

Fig. 2. Result of immunocytolocalization study showing the presence of ES (excretory/secretory) epitopes (arrows) on the tegument of the spiral canal (sp) of larval *Taenia solium*. Rabbit anti-serum against ES products from larval *T. solium* was used as the developing serum.

Human sera

Table 1 shows the results of testing ES and somatic antigens with sera samples from various groups of patients. Among the three different sources of somatic antigens, cystic fluid (F) yielded the largest number of false positive reactions. As compared with the somatic extracts, ES antigens yielded a smaller number of positive reactions with the sera from suspected cases. However,

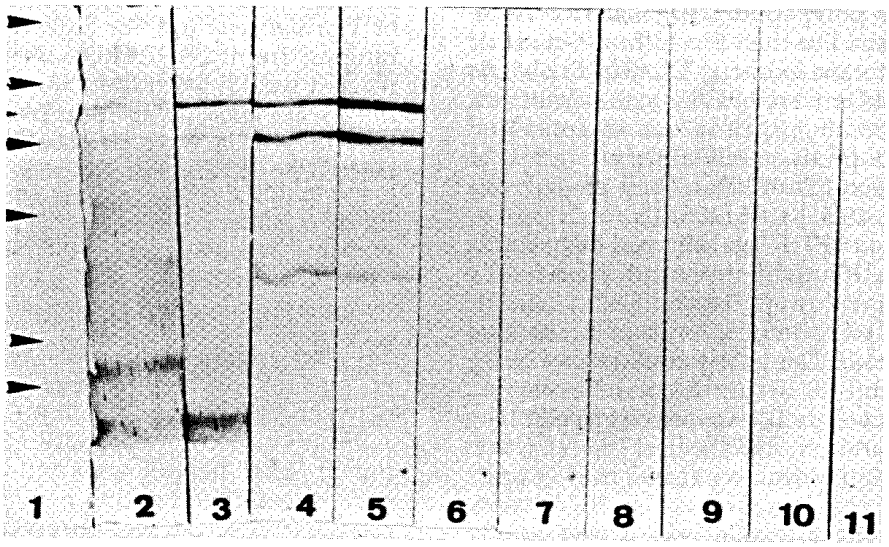

Fig. 3. Testing the cross-reactivities of excretory/secretory antigens by enzyme-linked immunotransfer blot (EITB). Rabbit antisera against various heterologous parasites were used as the developing serum. Note the absence of cross-reactions in lanes 6–11. Lane 1, standard marker: 26, 36.8, 48.1, 58, 84, 116 kDa (from bottom to top). Lane 2, larval *Taenia solium* W. Lane 3, larval *Taenia hydatigena* W. Lane 4, normal pig serum. Lane 5, normal pig muscle. Lane 6, *Hymenolepis diminuta*. Lane 7, *Dipylidium caninum*. Lane 8, *Fasciolopsis buski*. Lane 9, *Ascaris suum*. Lane 10, *Metastrongylus apri*. Lane 11, normal rabbit serum.

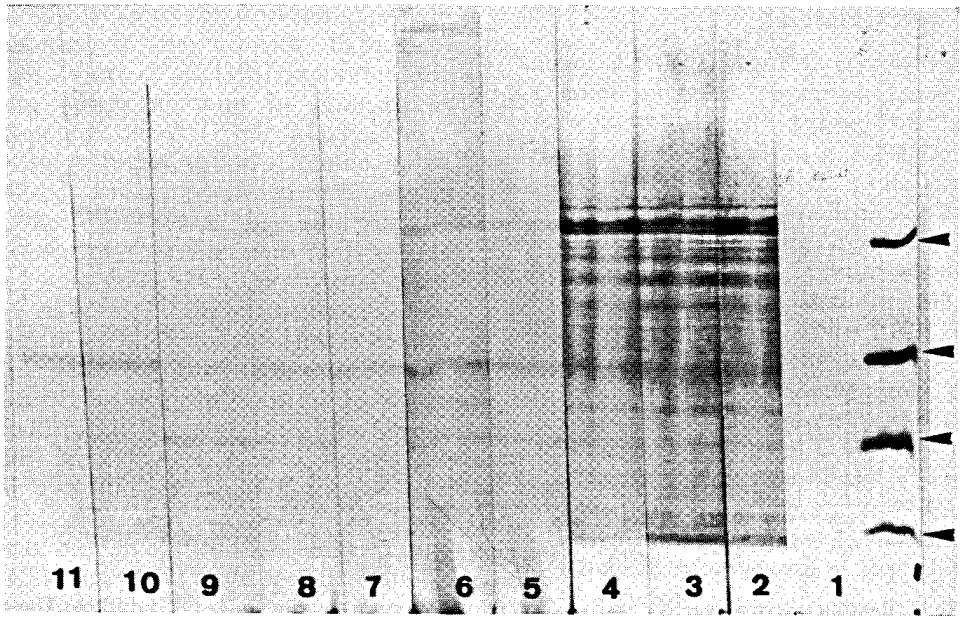

Fig. 4. Testing of the cross-reactivities of excretory/secretory antigens by enzyme-linked immunotransfer blot (EITB). Pig antisera against various heterologous parasites were used as the developing serum. Note the presence of strong cross-reactions in lanes 2–4. Lane 1, standard marker: 14.3, 20.1, 29, 39.8, 58.1, 97.4 kDa (from bottom to top). Lane 2, larval *Taenia solium* W. Lane 3, larval *Taenia solium* F. Lane 4, larval *Taenia hydatigena* F. Lane 5, *Trichinella spiralis*. Lane 6, *Trichuris suis*. Lane 7, *Metastrongylus apri*. Lane 8, *Fasciolopsis buski*. Lane 9, larval *Taenia solium* implant. Lane 10, normal pig serum.

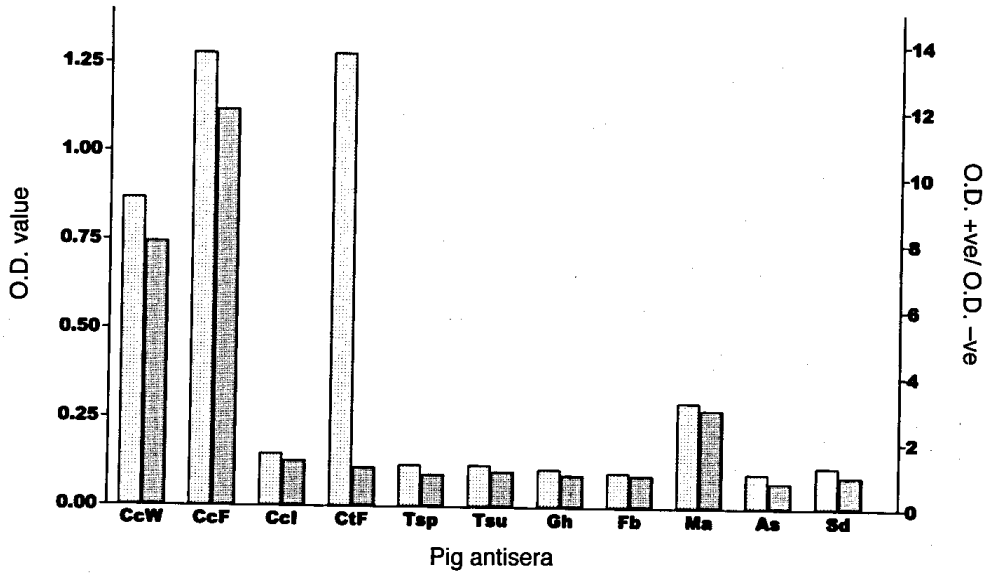

Fig. 5. Results of a cross-reactive study of excretory/secretory antigens by double antibody ELISA. The following rabbit antisera against common parasites of pigs were used as the developing serum: anti-larval *T. solium* (CcW), larval *T. solium* fluid (CcF), larval *T. solium* implant (CcI), larval *T. hydatigena* fluid (CtF), *Trichinella spiralis* (Tsp), *Trichuris suis* (Tsu), *Gnathostoma hispidum* (Gh), *Fasciolopsis buski* (Fb), *Metastrongylus apri* (Ma), *Ascaris suum* (As), *Stenphanurus dentatus* (Sd). The cut-off value was set at 0.6; ▨ O.D. (absorbance) value; ▤ O.D. + ve/O.D. –ve.

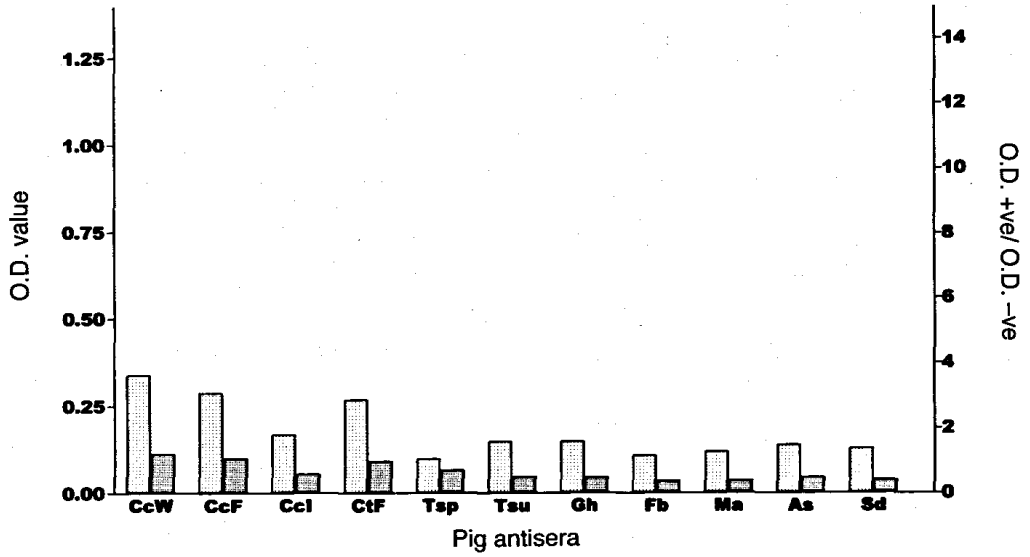

Fig. 6. Results of cross-reactive study of excretory/secretory antigens by FAST ELISA. The following rabbit antisera against common parasites of pigs were used as developing serum: anti-larval *Taenia solium* (CcW), larval *T. solium* fluid (CcF) larval *T. solium* implant (CcI), larval *T. hydatigena* fluid (CtF), *Trichinella spiralis* (Tsp), *Trichuris suis* (Tsu), *Gnathostsoma hispidum* (Gh), *Fasciolopsis buski* (Fb), *Metastrongylus apri* (Ma), *Ascaris suum* (As), *Stephanurus dentatus* (Sd). The cut-off value was set at 0.22; ▨ O.D. (absorbance) value; ■ O.D. +ve/O.D. –ve.

these antigens yielded no false positive reactions with heterologous antisera.

Discussion

The present study shows that it is possible to obtain ES antigens from the bladder worm stage of *T. solium*. Our experimental evidence indicates that the antigens recovered from the culture medium were true excretory/secretory products of the worms and not the degenerated components of the outer bladder wall. The worms remained active throughout the entire period of culture, as shown by the continuous inflation and deflation of the bladder. Sloughing of the bladder wall did not occur as there was no tissue debris in the culture medium. The immunocytolocalization study further confirmed the presence of ES epitopes on the tegument of the wall of the spiral canals.

The EITB results show that, unlike some helminths, specific immunodominant bands were absent in the ES products of larval *T. solium*. As in somatic antigens, they also contain too many conserved molecules. This would explain why ES antigens are not highly specific. Landa *et al.* (1994) isolated a glycoprotein enriched fraction from the somatic extracts of larval *T. solium* by affinity chromatography. Six prominent glycoproteins with M_r 180, 103, 96, 68, 55 and 45 kDa were located on the tegumental surface of the bladder wall. The 55 kDa band was found to correspond to the heavy chain of pig IgG. The last three bands with lower M_r observed by Landa *et al.* (1994) probably correspond with the 66, 58 and 43 kDa bands observed in the present study. The discrepancy is probably due to the different calibration method used.

However, it is not known whether the parasite synthesizes these molecules or acquires them directly from the host. In the present study, the host-like molecules, which are supposedly borne on the parasite surface, can actually be discharged into the ambient environment. This might imply that the molecules are not tightly anchored onto the external parasitic surface and they might be regularly replaced by the worm during metabolism. Nevertheless, further studies are required to determine the biological significance of such molecules *in vivo*.

Table 1. Comparison of the double antibody ELISA results using sera samples from patients with suspected cysticercosis or other parasitic diseases with the following antigens: whole cysts (W), membrane (M), fluid (F) and excretory/secretory (ES) products. The cut-off values for W, M, F and ES were set at 0.354, 1.578, 0.189 and 0.6 respectively.

	Mean OD value			
	W	M	F	ES
Cysticercosis				
Biopsy (1)*	0.358(1)†	1.752(1)	0.961(1)	0.663(1)
Calcified cysts (10)	0.153	0.563	0.193(3)	0.306(1)
Epilepsy (5)	0.118	0.585	0.09	0.258
Meningitis (20)	0.245(5)	0.843(4)	0.385(11)	0.52(6)
Hydatidosis (1)	0.405(1)	1.187	0.949(1)	0.371
Angiostrongyliasis (2)	0.196	0.006	0.009	0.031
Clonorchiasis (10)	0.044	0.036	0.025	0.038
Trichinosis (3)	0.097	0.033	0.16	0.033
Ascariasis (6)	0.126	0.094	0.072	0.073
Trichuriasis (3)	0.046	0.051	0.035	0.038
Gnathostomiasis (1)	0.134	0.15	0.018	0.051

* Number of cases; † Number of positive cases.

The presence of a host IgG molecule and serum protein have been documented in the somatic tissues of several metacestodes. Vido *et al.* (1987) reported the presence of human serum albumin in *Echinococcus granulosus* while Kamango *et al.* (1985), Machnicka & Grzybowski (1986) and Joshua *et al.* (1990) demonstrated a non-immunogenic bovine serum protein in the cystic fluid of *T. bovis*. Kwa & Liew (1978) suggested that the coating of the tegument of *T. taeniaeformis* metacestode with host-IgG proteins would enhance the survival of the parasite. Willms *et al.* (1986) also characterized two porcine IgG epitopes in larval *T. solium*. However, it is not known whether the parasite synthesizes these molecules or acquires them directly from the host. But their presence in the ES products suggests that the former origin is more likely. Nevertheless, further studies are required to determine the biological significance of such molecules.

For the diagnosis of human cysticercosis using ELISA, it appears that the ES antigens are more specific than the cystic fluid or cyst wall antigens. The fluid antigens cross-reacted with the antisera against hydatidosis and five other parasitic diseases. However, ES antigens are less sensitive than the somatic antigens because a substantially smaller number of cases with neurological manifestations was tested positive. For the diagnosis of porcine infections, however, ES antigens cross-reacted with the antisera against *T. hydatigena* and *M. apri* which are the common parasities of Chinese pigs. Therefore, ES antigens would not be suitable for immunodiagnosis if these two species are indigenous in pigs.

FAST-ELISA seems to be less sensitive but slightly more specific than the traditional double antibody ELISA. It yielded substantially lower OD values and OD^{+ve}/OD^{-ve} ratios even when the postive control sera were tested. Moreover, the high cost of the FAST-ELISA kit makes it less likely to be used for routine mass screening in abattoirs. Another major disadvantage is that washing of the beads with a spray container could easily lead to uneven washing and cross contaminations. However, Hancock & Tsang (1986) and Fagbemi & Hillyer (1990) reported satisfactory results when they used the assay to diagnose human schistosomiasis and dracunculiasis.

Several authors have reported that somatic glycoprotein antigens and EITB are good immunodiagnostic systems for both human and porcine cysticercosis (Tsang *et al.*, 1989; Gonalez *et al.*, 1990; Diaz *et al.*, 1992; Plancarte *et al.*, 1994). Tsang *et al.* (1989) extracted the glycoprotein antigens from the whole cysts by lentil-lectin chromatography. However, EITB is not a practical assay for large scale screening. Moreover, the actual location of the active epitope on the glycoprotein molecule has not been identified or characterized. Therefore, there is still an urgent need to search for a highly specific antigen and to develop a suitable assay for routine testing of pigs for cysticercosis.

Acknowledgements

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