

Trichinella spiralis: specificity of ES antigens from pre-encysted larvae

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ABSTRACT

Excretory/secretory (ES) antigens were obtained by culturing pre-encysted *Trichinella spiralis* larvae which were recovered from muscles of experimentally infected mice 14–15 days postinfection. Analyses of these antigens (PEL ES) with immunoblotting, SDS-PAGE and Triple Antibody ELISA showed that they yielded a low sensitivity and specificity when tested with antisera against the common nematodes of Chinese pigs. As compared to ES antigens from encysted larvae, PEL ES also contained more low molecular mass proteins.

KEY WORDS: *Trichinella spiralis*, pre-encysted larvae, ES antigens, specificity, Nematoda, immunology

INTRODUCTION

It is well known that excretory/secretory (ES) antigens are more specific than crude somatic antigens in the immunodiagnosis of many nematodoses. GAMBLE *et al.* (1988) observed that ES antigens obtained from encysted muscle larvae of *Trichinella spiralis* after 24 h in culture were highly specific when they were applied to screen pigs for trichinellosis in Yugoslavia. KO & YEUNG (1989) noted a similar specificity when the antigens were used to detect antibodies in Chinese pigs. Antigenic epitopes unique to *T. spiralis* were found in proteins having molecular mass 45–53 kDa. Besides *in vitro* culture, the specific antigens were also successfully isolated from crude worm extracts by the rotating ampholine horizontal method (KO & YEUNG, 1991). However, KO & YEUNG (1989) discovered that ES products from adult worms yielded markedly weaker reactions when tested against homologous anti-pig sera using Triple Antibody ELISA and immunoblotting. False positive reactions with anti-*Metastrongylus apri* serum were also observed. Adult ES antigens consisted of epitopes different from those of muscle larvae.

Therefore, it appears that ES products released by *T. spiralis* during various stages of development exhibit different antigenicity. Presumably the first-stage larvae, which have an exceptional growth rate, will actively release ES materials soon after entering muscle cells. The larvae can grow from about 100 µm to 1 mm in length in less than three weeks. During the intracellular development of the worms, the muscle fibres will undergo re-organization to form “nurse cells”. Recent studies by LEE *et al.* (1991) and DESPOMMIER *et al.* (1990) have demonstrated the presence of ES antigens in some of the hypertrophic nuclei of these specialized cells. The authors suggested that the secreted antigens may play a role in the modulation of the host genomic expression.

The present study was undertaken to determine whether the ES antigens obtained from pre-encysted larvae (PEL ES) were antigenically different from those of the encysted worms (EL ES). The usefulness of PEL ES antigens in serodiagnosis was also examined.

MATERIALS AND METHODS

Animals

The strain of *T. spiralis* used was originally isolated from a pig in Ontario, Canada and has since been maintained in both Wistar albino rats and ICR mice. Encysted larvae were recovered from mice 30 days postinfection by the standard pepsin digestion method. Pre-encysted larvae were recovered from the muscles of mice 14–15 days postinfection. The infected muscles were cut into fine pieces and then placed in 0.8% NaCl solution in a Baermann's funnel at room temperature. A lamp was placed near the funnel stem to provide a heat gradient. After 4–5 h, the larvae were collected and they were concentrated by centrifugation for five minutes at 2000 RPM and 4°C in a refrigerated centrifuge (Beckman GPR, table top model) for five minutes. The worms were washed five times in sterile saline and then checked under an inverted microscope for viability before being transferred to culture flasks.

Positive control serum samples were obtained by orally infecting five weaned piglets and six albino New Zealand rabbits with 10 000 each. The animals were bled and killed one and a half months after infection. The final worm burden of the pigs was about 20 larvae/g of muscle. Seven uninfected piglets served as the negative control. Hyperimmune serum against the common parasites of Chinese pigs (*Trichuris suis*, *Metastrongylus apri*, *Gnathostoma hispidum* and cysticerci of *Taenia solium* were produced in nine piglets and 11 rabbits by three intramuscular injections at weekly intervals using crude antigens and Freund's complete and incomplete adjuvants.

Antigens

Crude somatic antigens of *T. spiralis* were prepared by sonication of muscle larvae as described by Ko & YEUNG (1989). Crude antigens of heterologous species which were collected from pigs slaughtered in the Kennedy Town Abattoir, Hong Kong, were also prepared in a similar manner.

ES antigens were obtained by maintaining encysted and pre-encysted worms for 24 h in RPMI 1640 medium in an autoflow microprocessor-controlled CO₂ incubator with an infrared sensor (Nuair). The maintenance methods basically followed those of Ko & YEUNG (1989) and GAMBLE *et al.* (1983). For the culture of pre-encysted larvae, about 1000 worms were maintained in each canted neck 25 cm² culture flask (Costar). ES antigens were concentrated from the supernatant solution by a sample concentrator (Speed-Vac). The concentrated samples were desalted by gel filtration using Bio-Gel P6DG (Bio-Rad). Protein concentrations of the pooled samples were determined by a Protein Assay Kit (Bio-Rad) and the absorbance of the samples was read at 594 nm with a spectrophotometer (Milton Roy).

After 24 h in culture, usually less than 5% of the worms would appear inactivated or dead. Therefore, most antigens recovered from the culture medium were primarily ES products rather than somatic antigens released by dying worms (see GAMBLE *et al.*, 1988).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, immunoblotting and enzyme-linked immunosorbent assays

The details of these methods have been published elsewhere (Ko & YEUNG, 1989). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to LAEMMLI (1970) in a Mini-Protean II cell (Bio-Rad).

4 µg protein was electrophoresed in 10% gel at 100V for 1.5 h. The gels were stained with a silver staining kit (Pharmacia). A computer-controlled laser densitometer (Ultrascan XL, LKB) was used to scan the stained gel. For molecular mass determinations, Dalton Mark VIII (Sigma) was used.

Enzyme-linked immunotransfer blotting (EITB) was performed on nitrocellulose paper (0.45 µm, Schleicher & Schnell), following the method of TOWBIN *et al.* (1979). A mini-transblot cell (Bio-Rad) was used and the free binding sites were saturated with 3% gelatin in TBS. The anti-rabbit IgG horse-radish peroxidase conjugate (affinity purified, Sigma) was diluted to 1:2000 (v/v). 4-chloro-1-naphthol was used as the substrate. Biotinylated SDS-PAGE standards (Bio-Rad) were used for molecular mass determinations.

The Triple Antibody ELISA was performed according to KO & YEUNG (1989) and GAMBLE *et al.* (1983). Chequer-board titrations were used to determine the optimal conditions of the assay. Flat bottom polystyrene plates (Linbro, Flow Lab) were coated overnight at 4°C with antigens (0.1 µg PEL ES, 0.2 µg EL ES, 0.4 µg crude antigens in 100 µl/well) diluted in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6). Coated plates were washed three times with 0.9% NaCl and 0.05% Tween 20 using an automatic washer (Ultrawash, Dynatech) before the addition of the test serum (100 µl/well). After incubation for 2 h at 37°C and then washing, rabbit anti-porcine IgG (1:30 000 for crude and ES, 1:25 000 for PEL ES) was added. After a further incubation at room temperature for 2 h, the plates were washed before the addition of 100 µl goat anti-rabbit IgG horse-radish peroxidase conjugate (Sigma) (1:30 000 for crude and ES, 1:20 000 for PEL ES). The plates were incubated overnight at 4°C. They were washed once more before the addition of ortho-phenylenediamine (OPD). The absorbance was read by an automatic ELISA reader (MR 710, Dynatech) at 490 nm (test filter) and 410 nm (reference filter). Absorbance values greater than 5× the mean OD of the normal pool were considered as positive.

RESULTS

SDS-PAGE

Using a non-gradient gel, the SDS-PAGE profiles of the PEL ES, EL ES and crude antigens were shown to be different (Fig. 1). The PEL ES antigens were consisted of at least 20 bands; the molecular mass of the prominent bands were at 15–20 kDa. The bands at 45, 47 and 53 kDa were weakly stained. However, these bands which contained the epitope specific to *T. spiralis* were distinctive in the EL ES antigens which were also consisted of more proteins with larger molecular mass.

EITB

EITB was carried out with crude antigens and ES antigens from both pre-encysted and encysted worms. Various antisera against common parasites of Chinese pigs were used as developing sera (Fig. 2).

Lanes 2–7 show the blotting results of PEL ES. No bands were observed when anti-*G. hispidum* (lane 6) and *M. apri* (lane 7) sera were used as developing sera. Weakly stained bands were observed against the following antisera: four at 15, 18, 45 and 56 kDa against anti-*T. spiralis* serum (lane 3), one at 15 kDa against anti-*T. suis* (lane 4) and one at 56 kDa against anti-*C. cellulosae* (lane 5).

Lanes 8–19 show the blotting results of ES antigens from encysted worms and crude worm extracts. Intensively stained bands were present at 45, 47 and 53 kDa

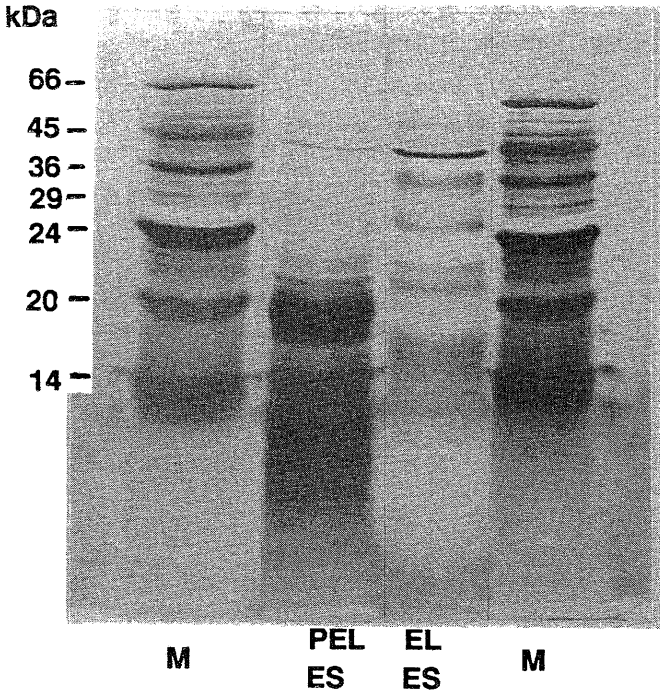


FIG. 1. SDS-PAGE electrophoresis using non-gradient gel to compare ES antigens from pre-encysted (PEL) and encysted larvae (EL). PEL antigens were dominated by low molecular mass proteins (15–20 kDa). Proteins with specific epitopes (45, 47, 53 kDa) were prominent in EL ES. M=molecular mass marker.

when anti-*T. spiralis* serum was used to blot both EL ES and crude antigens (lane 8 and 10). Three faint bands at 59, 41, 33 kDa were observed in lane 14 ES antigens from encysted larvae and anti-*T. suis* were used. However, at least 10 strong bands were shown when crude antigens were blotted with the same antiserum (lane 18). The crude antigens also cross-reacted with anti-*G. spinigerum* and *C. cellulosa* serum (lanes 16, 17).

ELISA

Sera samples, which were taken from experimentally infected mice on days 14 and 30 postinfection, were tested by Triple Antibody ELISA against crude, EL ES and PEL ES antigens. PEL ES antigens yielded negative reactions. The OD +ve/OD -ve ratios were as follows: for crude antigens, 4.6, 8.7; for EL ES antigens, 5.9, 12; for PEL ES antigens, 0.49, 0.79.

Fig. 3 compares the sensitivity of the different antigen when they were tested against pig anti-*T. spiralis* serum. Both the conjugates and substrates were under excess condition. For PEL ES antigens, the increase in OD values reaches the maximum at 12 $\mu\text{g/ml}$ whereas for EL ES and crude antigens, the increase reaches the maximum at 1 $\mu\text{g/ml}$ and 12 $\mu\text{g/ml}$ respectively.

Fig. 4 compares the specificity of the different antigens when they were tested against various pig antisera. PEL ES antigens exhibited a low specificity. They produced a substantially low OD+ve/OD-ve ratio when tested against the

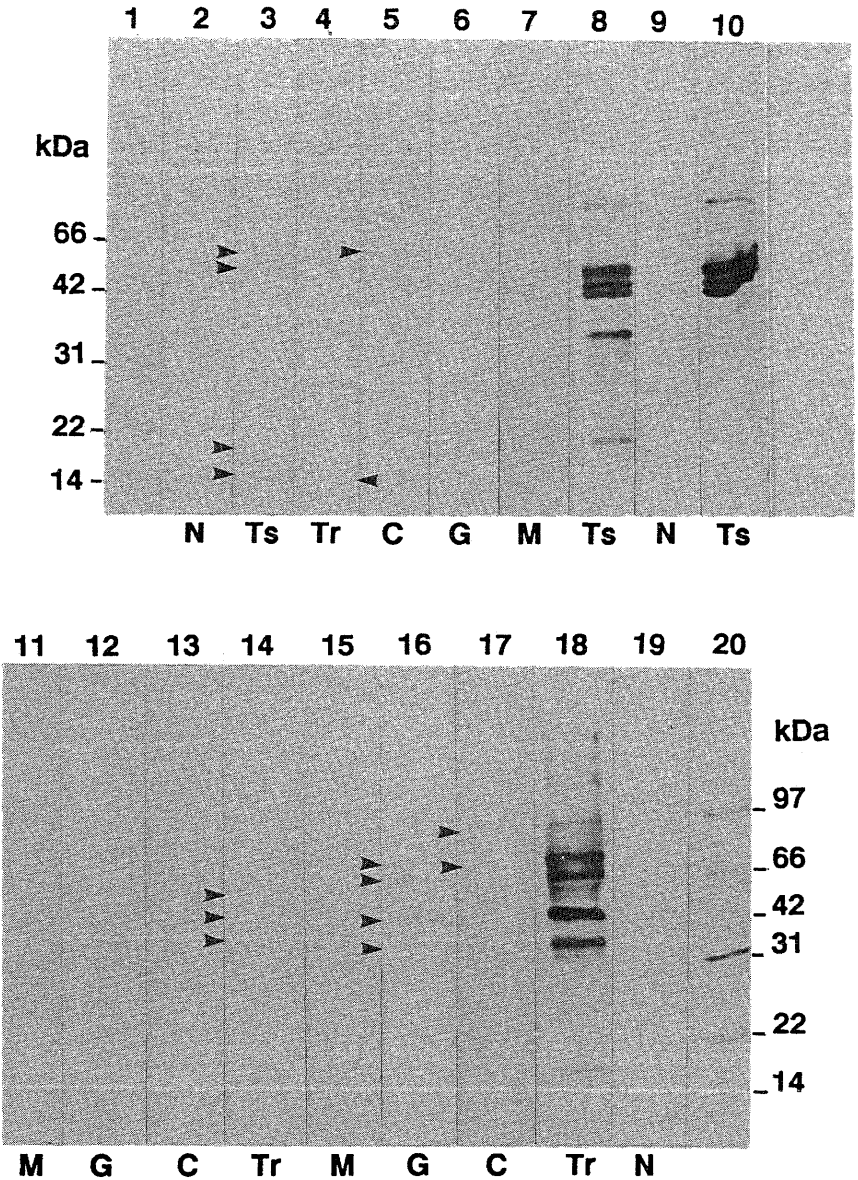


FIG. 2. Enzyme-linked immunotransfer blot of crude and ES antigens from pre-encysted (PEL) and encysted larvae (EL), using homologous and heterologous rabbit antisera as developing serum. Molecular mass marker: lanes 1 and 20. PEL ES antigens: lanes 2-7. EL ES antigens: lanes 8-9 and 11-14. Crude antigens: lanes 10 and 15-19. The following antisera were used: normal pig serum (N), *Trichinella spiralis* (Ts), *Trichuris suis* (Tr), *Cysticercus cellulosae* (C), *Gnathostoma hispidum* (G), *Metastrongylus apri* (M). Arrows indicate positions of weakly stained bands.

homologous anti-*T. spiralis* serum; the ratio was below the $5\times$ mean OD-ve of control samples, i.e. a false negative reaction. However, they yielded a false positive reaction with anti-*M. apri* serum. As compared to EL ES antigens, PEL ES antigens yielded higher OD+ve/OD-ve ratios against anti-*G. hispidum* and *C. cellulosae* sera.

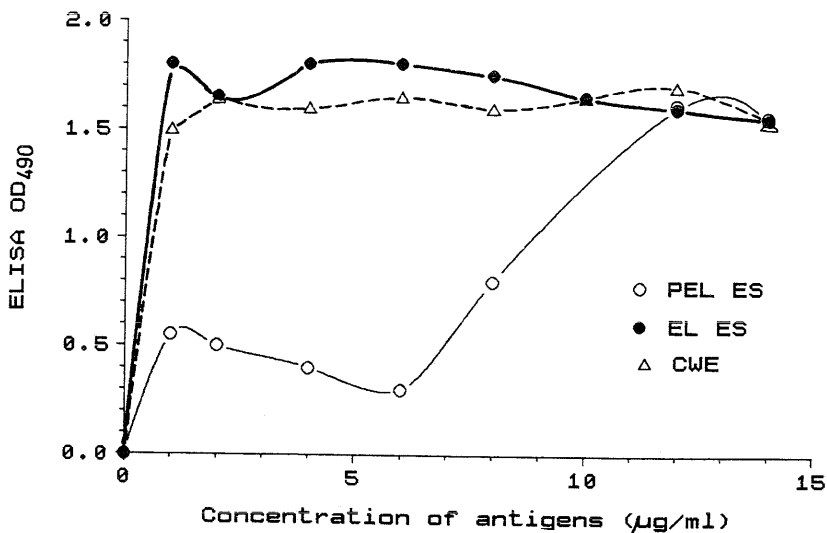


FIG. 3. Comparison of OD readings (Triple Antibody ELISA) at various concentrations of crude worm extract (CWE) and ES antigens from encysted (EL) and pre-encysted larvae (PEL). Dilution of pig anti-*Trichinella spiralis* serum was 1:10 and conjugate 1:2000.

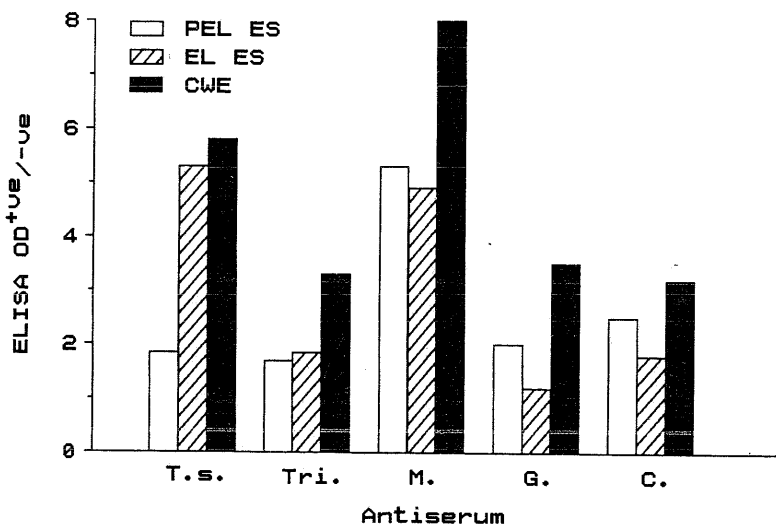


FIG. 4. The OD+ve/OD-ve ratios obtained under optimal conditions in Triple Antibody ELISA when crude and ES antigens from encysted (EL) and pre-encysted larvae (PEL) were tested against the following antisera produced in pigs: *Trichinella spiralis* (Ts), *Trichuris suis* (Tr), *Metastrongylus apri* (M), *Gnathostoma hispidum* (G) and *Cysticercus cellulosae* (C).

DISCUSSION

Data in the present study demonstrate that the ES products from pre-encysted and encysted larvae are antigenically different. The former, which are dominated by low molecular mass proteins, have a low sensitivity and specificity. Only weakly stained bands were obtained in the blotting with anti-*T. spiralis* serum. These antigens also invariably yielded low OD positive to negative ratios when tested against homologous antiserum by the Triple Antibody ELISA. The lack of specificity of the antigens was indicated by the strong false positive reaction against anti-*M. apri* serum. EITB also showed some cross-reactivities with anti-*T. suis* and

C. cellulosae sera. Therefore, the PEL ES antigens are unsuitable as serodiagnostic antigens. The lack of specificity is probably due to the fact that the immunodominant 45–53 kDa proteins of the EL ES antigens are only present in small quantities in PEL ES antigens. GAMBLE & GRAHAM (1984) reported that the antigenic epitope unique to *T. spiralis* was recognized by an IgM monoclonal antibody (7C₂C₅). However, our studies have shown that the epitope could also be recognized by two IgG₁ as well as IgM monoclonal antibodies (unpublished data).

The antigenic profile of PEL ES products seems to resemble that of ES antigens obtained from adult worms as reported in an earlier study (KO & YEUNG, 1989). The adult worm ES antigens were found to yield weaker reactions in Triple Antibody ELISA. They were rich in low molecular mass proteins and lacked the specific 45–53 kDa bands (as demonstrated by EITB). A strong false positive reaction with anti-*M. apri* serum was also observed. But, as compared to PEL ES antigens, those from the adult worms contained more high molecular mass proteins. Therefore, it appears that the composition of the ES products released by *T. spiralis* will vary according to the stage of worm development in the host.

The specific proteins are probably secreted by the stichosome of this trichuroid nematode in substantial quantities during the active phase of encystment. The transformation of the striated muscle fibre of the mammalian host into a nurse cell with hypertrophic nuclei is completed by 20–30 days postinfection. In a recent study using immunocytochemical methods and monoclonal antibodies as probes, we observed that some hypertrophic nuclei of nurse cells at 30 days postinfection were stained positively for EL ES antigens. It was suggested that only some molecules in the ES pool can enter the nuclei or different monoclonal antibodies may have different affinities to the subunits of the glycoproteins which can enter the nuclei (LEE *et al.*, 1991).

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