

# PRODUCTION SPECIFIC ANTIGENS FROM *TRICHINELLA SPIRALIS* USING A CONTINUOUS ELUTION METHOD AND ISOELECTRIC FOCUSING

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**Abstract.** Immunodominant antigens of 45-53 kDa (one band per fraction) were obtained from excretory/secretory (E/S) and somatic products of infective-larvae of *Trichinella spiralis* using a continuous-elution method. They were further resolved by isoelectric focusing into different isoforms (45kDa : pI4.47, 5.09, 5.47 and 5.86; 47kDa : pI4.72 and 4.97; 53kDa : pI4.86, 5.11, 5.44 and 5.78). In immunoblotting, the isoforms of pI 5.09, 5.86, 4.97, 5.44 and 5.78 did not cross-react with antisera against *Trichuris suis*, *Metastrongylus apri*, *Gnathostoma hispidum* and *Stephanurus dentatus*. Hence, they have the potential to serve as specific antigens for the serodiagnosis of trichinellosis.

## INTRODUCTION

Trichinellosis is common in Hong Kong (Ko, 1984) and in many parts of China (Xu, 1975). However, an efficient method for producing diagnostic antigens from the parasite is still lacking. Pigs imported from China are the major source of pork consumed in Hong Kong (Ko, 1984). Nevertheless, they are commonly infected with *Trichinella spiralis*, *Trichiuris suis*, *Metastrongylus apri*, *Gnathostoma hispidum* and *Stephanurus dentatus*. (Chan and Ko, 1990). Therefore, before an effective serodiagnostic system can be developed, cross-reacting antigens between the species must be removed. In this project, attempts were undertaken to isolate the 45-53kDa antigens from the crude and E/S products using a newly developed continuous-elution method. The antigens were further resolved using IEF to highly specific isoforms suitable for screening of Chinese pigs.

## MATERIALS AND METHODS

*T. spiralis* (Canadian strain) which was originally isolated from pigs in Guelph, Ontario in 1967 was used in the present study. The stock was maintained by consecutive passage of muscle larvae through Wistar rats and ICR mice at 6-7 months intervals. Muscle larvae were harvested by digestion of the minced muscles in 0.6% (v/v) pepsin (Sigma) /1% (v/v) HCl (Merck) at 37°C for 4 hours. E/S products were obtained by culturing the infective larvae for 24 hours at 37°C

and 5% CO<sub>2</sub> in 250 ml canted-neck culture flasks (Coaster), containing RPMI 1640 medium (Gibco-BRL), penicillin (100 IU/ml) and streptomycin (100 µg/ml). The supernatant containing the E/S products was recovered and concentrated by ultrafiltration using a stirred cell (Model 8200, Amicon). Crude worm extracts were prepared by sonicating (W-385 Heat Systems-Ultrasonics) infective larvae in PBS and then incubating the suspension for 24 hours at 4°C. The soluble worm extracts were obtained by ultracentrifugation of the suspension for 45 minutes at 25,000 rpm and 4°C. Protein concentrations were determined by a Protein Assay Kit (Bio-Rad). The absorbance of the samples was measured with a spectrophotometer at 595 nm (Milton Roy MR-710).

## Continuous-elution SDS-PAGE

Continuous-elution SDS-PAGE was performed using the Prep-Cell (Model 491, Bio-Rad). The discontinuous buffer system was used. A cylindrical column of polyacrylamide (Kodak) gel was prepared using a 28 mm ID casting tube. It consisted of an upper 4% stacking and a lower 12% separating gel. Their heights were 2 and 7 cm respectively. The casting tube was assembled with the upper and lower buffer chamber. The latter was connected to an external recirculation pump (Bio-Rad). Samples were prepared by mixing 10 mg of either E/S products or crude worm extracts with equal volume of the sample buffer (two-fold concentrate) (125 mM Tris-HCl, pH 6.8; 20% (v/v) glycerol; 4% (w/v) SDS; 5% (v/v)

$\beta$ -mercaptoethanol and 0.002% bromophenol blue). 2.5 ml sample (4 mg/ml) was heated for 5 minutes at 95°C before being loaded onto the stacking gel. The recirculation pump was operated at 100 ml/minutes.

The samples were electrophoresized for 10 hours at 500 V, 40 mA and 20 W (3000xi power supply, Bio-Rad). Resolved proteins were collected at the base of the gel column using a dialysis membrane with 5 kDa cut-off. They were continuously eluted at 1 ml/minutes from the column using an external peristaltic pump (Econo-Pump, Bio-Rad). The absorbance of the eluant at 595 nm was monitored using a UV monitor (Bio-Rad). The cut-off absorbance was 0.05 OD and fractionated proteins were retrieved using a programable fraction collector (Model 2110, Bio-Rad). To determine the optimal conditions for the purification, the experiment was repeated by varying the height of the separating gel. Alternatively, the elution rate or the sample loading was changed. The protein contents eluants were determined using a Detergent-compatible Protein Assay Kit (Bio-Rad).

#### Analytical SDS-PAGE

The eluants were analyzed by slab-gel SDS-PAGE using the Mini-Protean II Cell (Bio-Rad). Polyacrylamide gels of 12% separating and 4% stacking zones were cast using gel cassettes provided with the cell. Samples and molecular weight markers (MW-SDS-70L, Sigma) were diluted 1/4 using sample buffer. They were electrophoresized for 1.5 hours at 120 V constant voltage (200/2 power supply, Bio-Rad). The gel was either fixed and then stained using silver staining (Pharmacia), or subjected to immunoblotting.

#### Immunoblotting

Immunoblotting was performed according to the method of Towbin *et al* (1979) with some modifications. A mini-transblot cell (Bio-Rad) was used. After analytical electrophoresis, the gel was equilibrated for 30 minutes with a 4°C transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3). Nitrocellulose membranes (0.45  $\mu$ m pore size, Scheicher and Schnell), filter papers (Whatman No.1) and fiber pads were saturated for 30 minutes in the same buffer. Transfer cassettes were assembled according to the operating instructions by Bio-Rad. Transfer was carried out from cathode to anode at 110 V constant

voltage (250/2.5 power supply, Bio-Rad) for 3 hours. The paper strips with transferred proteins were exposed to 5 ml of anti-*T. spiralis* serum diluted 1/100 in PBS overnight. Antisera against *T. suis*, *M. apri*, *G. hispidum* and *S. dentatus* were used as the heterologous antibodies. The strips were then incubated with Protein A horse-radish peroxidase (HRP) conjugate (Bio-Rad) diluted 1/2,000 for 3 hours before developed using 0.3% (w/v) 4-chloro-1-naphthol (Sigma) as substrate. 6B (Sigma) was used as the molecular weight markers and was treated by streptavidin-HRP (Cappel) before development. Normal pig serum served as the negative control.

#### Isoelectric focusing

Isoelectric focusing was performed using the Rotorfor Cell (Bio-Rad). The cooling finger of the horizontal focusing column was connected to an external refrigerated recirculator (Model 4860, Bio-Rad). 55 ml of distilled deionized water containing 2% ampholytes of pI 3-10 or 4-6.5 (Pharmacia) were prefocused for 1 hour at 12 W constant power (3000xi, Bio-Rad). 0.5 mg of the 45-53 kDa antigens were then focused for 6 hours at 12 W. 10 mg of either crude worm extracts or E/S products were also fractionated for comparison. Twenty fractions obtained in each run were collected under reduced pressure. They were analyzed by PhastGel IEF (pI 3-10) using the Phast System (Pharmacia). The pI's of fractions were also recorded with a microelectrode (PHM-82 radiometer, Copenhagen). The specificity of the isoforms was determined using immunoblotting.

## RESULTS

#### Continuous-elution SDS-PAGE

The molecular masses (MM) of proteins in the E/S products varied from 8-113 kDa. The majority of them ranged from 30-60 kDa and the ones of 42 kDa were most abundant (*ie* 6% of the total yield). Six high MM (*ie* > 60 kDa) proteins were detected. Proteins of 66, 73, 75 and 84 kDa were present in low quantities (*eg* <1%) whereas 97 and 113 kDa proteins were numerous (*ie* 4%). A similar profile was obtained from the crude extracts. The yield for both samples was about 82%.

The efficiency and resolution of electrophoresis varied to height of separating gel column, protein and elution rate. Using a 7 cm gel, the run was completed in 8 hours and one band per lane was obtained. Proteins with a mass difference of 2 kDa were clearly resolved (Fig 1). The use of a 8 cm column prolonged the run by 2 hours and reduced the resolution. A 6 cm gel shortened the run but an incomplete separation of proteins was noted (Fig 2). An increase in the sample loading from 10 to 15 mg increased the yield from 8.7 to 12.9 mg. In both cases, sharp and distinct bands as those obtained in Fig 1 were obtained. However, loadings above 20 mg drastically lowered the resolution (Fig 3). An elution rate of 2 ml/minute yielded an 8 hours run a good resolution (Fig 1). At 3 ml/minute electrophoresis was shortened to 5 hours but the resolution was adversely affected.

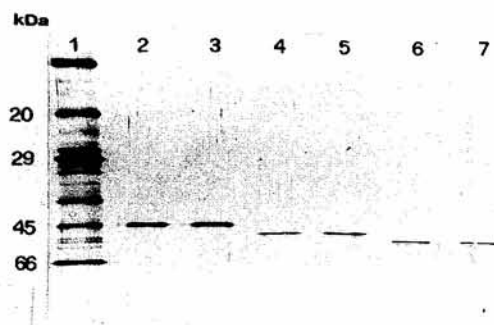


Fig 1 - Silver staining showing SDS-PAGE profiles of fractions containing 45 (Lanes 2, 3), 47 (Lanes 4, 5) and 53 kDa (Lanes 6, 7) proteins obtained from Prep-Cell. Lane 1: molecular weight markers.

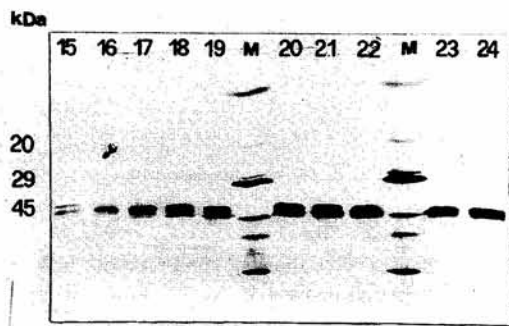


Fig 2 - Silver staining showing SDS-PAGE profiles of fractions 15-24 from Prep-Cell. Note the incomplete separation of proteins in fractions 18-22. Lanes M: molecular weight markers.

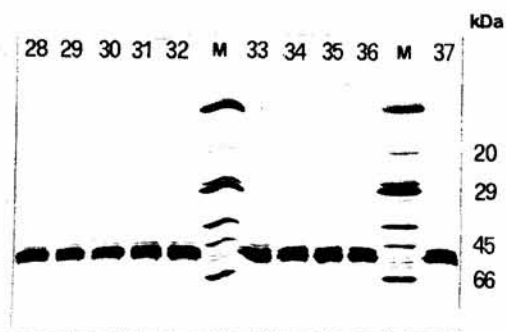


Fig 3 - Silver staining showing SDS-PAGE profiles of fractions 28-37 from Prep-Cell. Note the incomplete separation of proteins in these fractions. Lanes M: molecular weight markers.

### Isoelectric focusing

Four isoforms of pI 4.47, 5.09, 5.47 and 5.86 were identified from the 45 kDa proteins whereas two (pI 4.72 and 4.97) and four (pI 4.86, 5.11, 5.44 and 5.78) isoforms were detected from the 47 and 53 kDa proteins respectively. Fractionation using an ampholyte solution with pI 4-6.5 instead of 3-10 failed to affect the results. Similar data were obtained for both crude worm extracts and E/S products.

The yield was about 90% for both crude extracts and E/S products. The pI's of proteins from the E/S products ranged from 2.75-9.21 (Fig 4). The majority of them were focused between pI 4.21-6.87 although a distinct at pI 9.21 was also observed. Few proteins were found in the low pI (*ie* 2-4) region. Analytical SDS-PAGE showed that the 45- 53 kDa proteins were scattered in fractions 6-12 (pI 4.12-6.03).

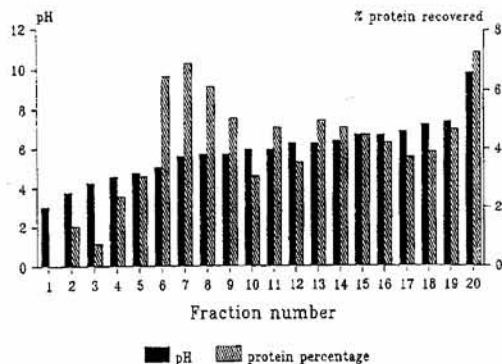


Fig 4 - pI profile of E/S products after IEF. A total of 8.92 out of 10 mg loading proteins was recovered.

A similar pattern was observed in crude worm extracts. However, they yielded less high pI (ie 9-11) proteins than the E/S products. When the focusing range was reduced from pI 3-10 to 4-6.5, the 45-53 kDa proteins were dispersed in fractions 5-15.

### Immunoblotting

Cross-reactions were observed between unpurified E/S products and antisera against anti- *T. suis*, *G. hispidum*, *M. apri* and *S. dentatus* sera. The strongest reaction was detected with anti- *T. suis* serum (Fig 5); proteins of 10-72 kDa were recognized. Five distinct bands of 66, 47, 39, 31 and 14 kDa were seen. Four bands (66, 39, 30 and 14 kDa) were highlighted with anti- *M. apri* serum. Antisera against *S. dentatus* and *G. hispidum* reacted weakly with the E/S products; faint bands of 43 and 60-49 kDa respectively were obtained.

After purification by Prep-Cell, the 45 kDa proteins still cross-reacts with all the antisera except that of *G. hispidum* (Fig 6a). Similar results were obtained for the 47 kDa proteins except the intensity of the signals were weaker (Fig 6b). The 53 kDa molecules were the most specific when compared to the 45 and 47 kDa proteins. They only cross-reacted with anti-*T. suis* serum (Fig 6c).

However, using IEF, cross-reactions between two isoforms (pI 5.09 and 5.86) from the 45 kDa molecules and the heterologous antisera were successfully eliminated (Fig 7) although the pI 4.47 and 5.47 isoforms still reacted with anti- *T. suis* serum. Specific isoforms (pI 4.97; pI 5.44 and 5.78) were also obtained from the 47 and 53 kDa proteins. However, they yielded less distinct signals with the antiserum of *T. spiralis* than those from the 45 molecules (Fig 8).

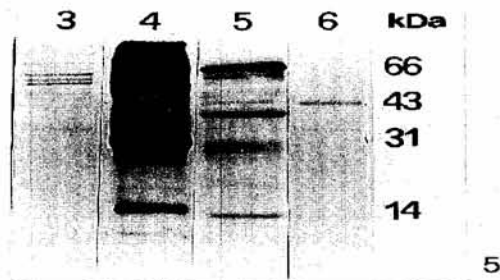


Fig 5 - Immunoblots showing cross-reactions between E/S products and anti-*G. hispidum* (Lane 3), *T. suis* (Lane 4), *M. apri* (Lane 5) and *S. dentatus* (Lane 6) sera.

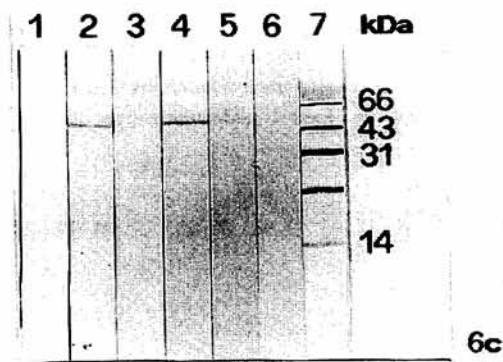
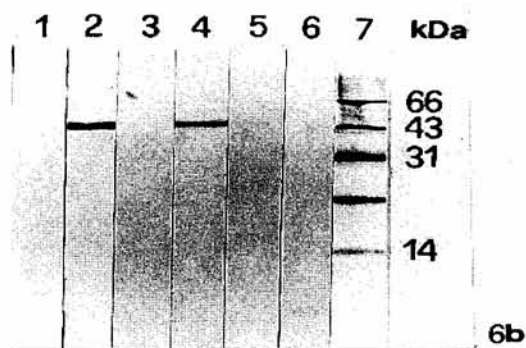
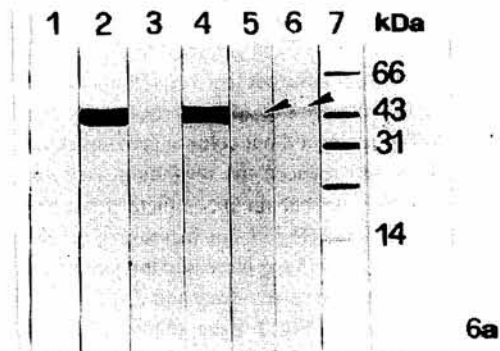


Fig 6 - Immunoblots showing cross-reactions between Prep-Cell purified 45 (a), 47 (b) or 53kDa (c) proteins and various pig antisera.

Lanes: 1. Normal pig serum; 2. Anti- *T. spiralis* serum; 3. Anti- *G. hispidum* serum; 4. Anti- *T. suis* serum; 5. Anti- *M. apri* serum; 6. Anti- *S. dentatus* serum; 7. Molecular weight markers.

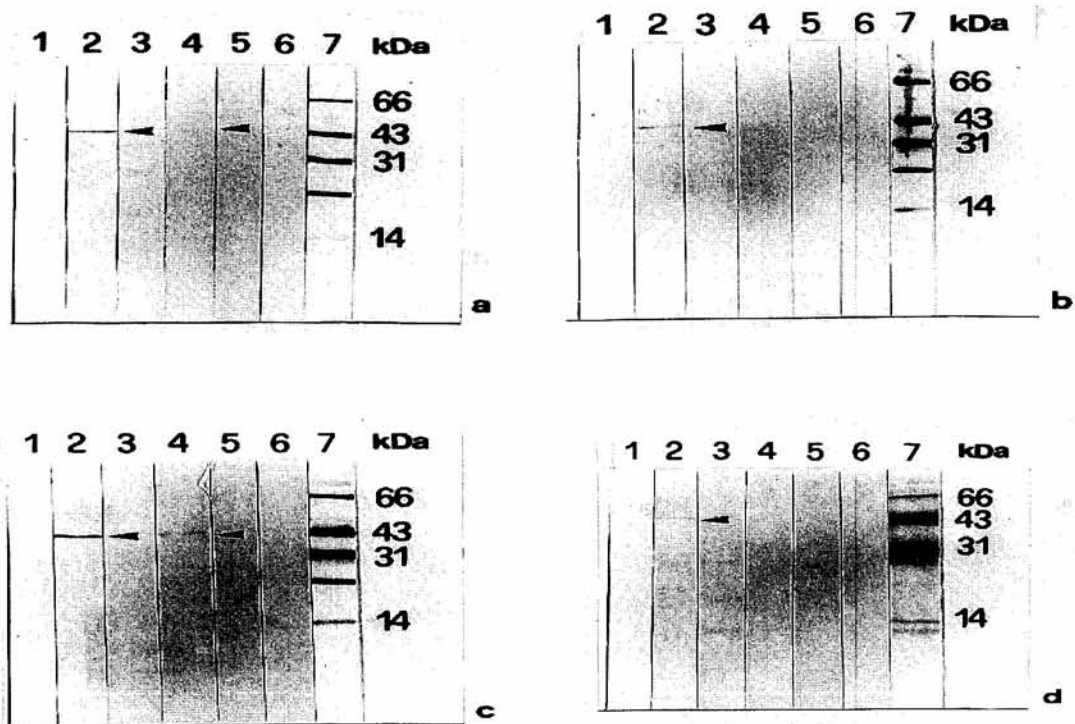


Fig 7 - Immunoblots showing cross-reactions between four isoforms from 45kDa proteins (pI 4.47: a; pI 5.09: b; pI 5.47: c; pI 5.86: d) and various pig antisera.

Lanes: 1. Normal pig serum; 2. Anti- *T. spiralis* serum; 3. Anti- *G. hispidum* serum; 4. Anti- *T. suis* serum; 5. Anti- *M. apri* serum; 6. Anti- *S. dentatus* serum; 7. Molecular weight markers.

## DISCUSSION

### Continuous-elution SDS-PAGE

Sample loading, internal diameter and height of gel column, and elution rate are the most important parameter of this technic. A combination of a sample loading of 5 mg, a gel column of mm ID (4% stacking and 12% separating gel of 2 and 7 cm in height, respectively) and an elution rate of 2 ml/minute provided the optimal conditions for fractionation.

The resolution in SDS-PAGE is directly proportional to the distance of migration of proteins (Garfin, 1990). Nevertheless, it may diminish when the electrophoresis distance exceeds certain optimal values. It is probably because of the prolonged interactions between polyacrylamide and the samples.

The net yield was increased by 48% when the sample loading was changed from 10 to 15 mg. Loading above 20 mg, however, adversely affected the resolution although the yield was further improved. Since the poor resolution could not be rectified by varying the height of the stacking gel, the diameter of the gel may be the limiting factor in this experiment. Therefore, gel columns of large ID (*ie* 37 mm) are more suitable for high sample loading (*ie* > 20 mg). The efficiency of the run was enhanced by raising the elution rate from 1 to 2 ml/minute. At 3 ml/minute however, neighbouring fractions tended to overlap with each other. This may be due to a mixing of proteins on the dialysis membrane.

Similar yields were obtained from both E/S products and crude worm extracts. Since the latter is easily available in substantial quantity, they can serve



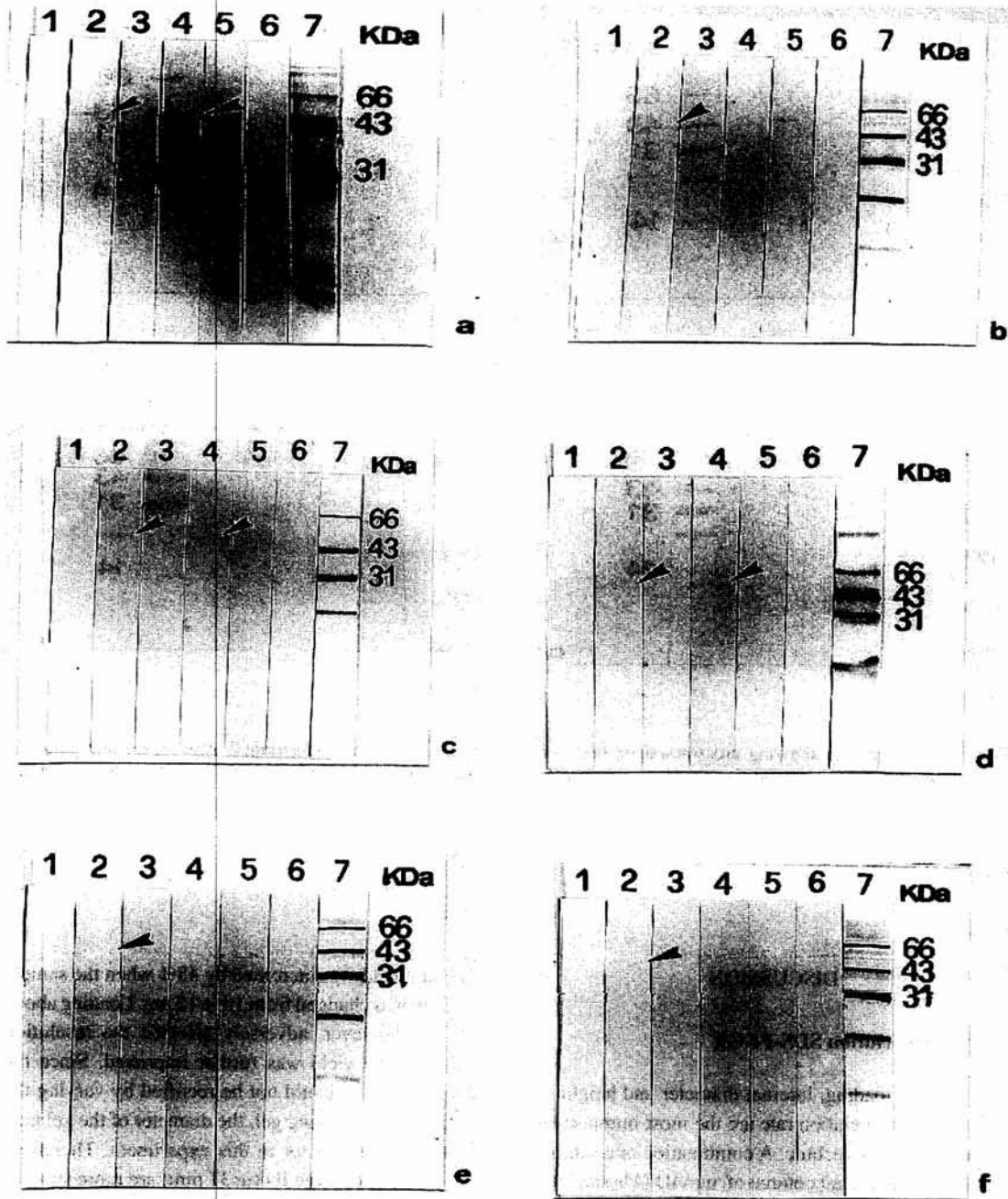


Fig 8 - Immunoblots showing cross-reactions between isoforms from 47kDa proteins (pI 4.72: a; pI 4.97: b) or 53kDa proteins (pI 4.86: c; pI 5.11: d; pI 5.44: e; pI 5.78: f) and various pig antisera.

Lanes: 1. Normal pig serum; 2. Anti- *T. spiralis* serum; 3. Anti- *G. hispidum* serum; 4. Anti- *T. suis* serum; 5. Anti- *M. apri* serum; 6. Anti- *S. dentatus* serum; 7. Molecular weight markers.

as a better source of materials for purification.

Continuous-elution SDS-PAGE provides a one-step method to produce the 45-53 kDa proteins. The antigens are continuously eluted from the gel column and are prevented from mixing. Conventional purification methods are usually either multi-steps (Despommier *et al*, 1990; Mizuno, 1990) or are incapable of fractionating the immunodominant antigens efficiently (Gamble and Graham, 1984; Arriaga *et al*, 1989). Chromatofocusing requires a series of washing of the eluants to remove polybuffer before they can be used for further experiments (Despommier *et al*, 1990). In flatbed IEF, the antigens identified on polyacrylamide gels have to be first extracted. They are then deionized to remove ampholytes before they can be used in immunoaffinity chromatography is antibody-specific. This may cause a mixed elution of the antigens which share common epitopes (Gamble and Graham, 1984; Arriaga *et al*, 1989).

However, the limitation of the continuous-elution technique is the binding of SDS to the purified products. Since SDS is a potent protein-denaturing agent, it may affect the biological functions of the antigens. An effective measure to remove SDS from proteins is still lacking. Although ion-exchange chromatography (eg AG 11-A8, Bio-Rad) has been reported to be successful in displacing SDS from proteins, its effectiveness has yet to be determined.

### Isoelectric focusing

Although isoforms are commonly found in enzymes (Buckingham *et al*, 1992), they may also be associated with molecules involved in gene regulations (Muntoni *et al*, 1995) and muscle development (Hailstones and Gunning, 1990). The presence of isoforms in the 45-53 kDa molecules may have two implications: (1) some may be involved in modulating the development of host muscles. (2) others may play a role in the normal development of the larvae itself. The production of isoforms is also favorable for gene economy (Strachan, 1992). This is particularly important in *T. spiralis* which has a relatively small genome as compared to those of higher eukaryotes (Uitterlinden and Vijg, 1994).

IEF is less effective than continuous-elution SDS-PAGE in isolating the 45-53 kDa antigens from the E/S products and crude worm extracts. Since IEF

separates proteins on the basis different pI's, the antigens tend to be dispersed in several fractions, instead of being concentrated into distinct regions as in SDS-PAGE. However, the results show that the former can serve as a second purification step to supplement the latter.

### Immunoblotting

Of the five specific isoforms observed, the pI 5.09 and 5.86 isoforms from the 45 kDa proteins gave the strongest signals with anti-*T. spiralis* serum. The absence of bands with other antisera could not probably be due to an insufficient amount of antigens due to the following reasons. All lanes were loaded with an equal amount of the antigens. The results were not affected even though the concentration of the antisera was increased.

After IEF, most cross-reactive antigens were eliminated from the isoforms. However, some isoforms still reacted weakly with anti-*T. suis* serum. This indicates that *T. spiralis* and *T. suis*, which are closely related phylogenetically (Despommier and Muller, 1976), may share some epitopes differing in several amino acid residues.

### ACKNOWLEDGEMENTS

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### REFERENCES

- Arriaga C, Muniz E, Morilla A, Ortega-Pierres G. *Trichinella spiralis*: recognition of muscle larva antigens during experimental infection of swine and its potential use in diagnosis. *Exp Parasitol* 1989; 69: 363-72.
- Buckingham M, Houzelatein D, Lyons G, Ontell M, Oh MO, Sasson D. Expression of muscle genes in the mouse embryo. *Sym Soc Exp Biol* 1992; 46: 203-17.
- Chan SW, Ko RC. Cross-reactions between crude antigens of *Trichinella spiralis* and other nematodes of Chinese pigs. *Trop Biomed* 1990; 7: 143-50.
- Despommier DD, Gold AM, Buck SW, Capo V, Silberstein D. *Trichinella spiralis*: secreted antigens of the infective L1 larvae localize to the cytoplasm and nucleoplasm of infected host cells. *Exp Parasitol* 1990; 71: 27-38.

- Gamble HR, Graham CE. Monoclonal antibody-purified antigen for the immunodiagnosis of trichinosis. *Am J Vet Res* 1984; 45: 67-74.
- Garfin DE. One-dimensional gel electrophoresis. In: Deutscher MP, ed. *Guide to Protein Purification, Methods in Enzymology*. New York: Academic Press 1990; 182: 425-37.
- Hailstones DL, Gunning PW. Characterization of human myosin light chains and 1.3 and 3nm : implications for isoform evolution and function. *Mol Cell Biol* 1990; 10: 1095-104.
- Ko RC. Are there any parasitic disease problems in Hong Kong? In : Ko RC, ed. *Current Perspectives in Parasitic Diseases Proceedings of the Southeast Asian Symposium on Parasitology and Medicine held in Hong Kong Dec. 9-12, Hong Kong: University of Hong Kong Press, 1984: 29-51.*
- Mizuno N. Immunological studies on *Trichinella spiralis* infection with emphasis on time course studies of antibody responses against muscle larvae. *J Nara Med Assoc* 1990; 41: 651-69.
- Muntoni F, Melis MA, Ganau A, Dubowitz V. Transcription of the dystrophin gene in normal tissues and in skeletal muscles of a family with x-linked dilated cardiomyopathy. *Am J Hum Gene* 1995; 56: 151-7.
- Strachan T. In: Read AP, Brown T, eds. *The Human Genome*. UK: Information Press, 1992.
- Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc Natl Acad Sci USA* 1979; 76: 4350-4.
- Uitterlinden AG, Vijg J. *Two-dimensional DNA Typing - A Parallel Approach to Genome Analysis*. New York: Ellis Horwood Press, 1994.
- Xu N. *Animal Nematology*. Beijing: Scientific Publication, 1975.