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CROSS-REACTIONS BETWEEN *Toxocara canis* AND *Ascaris suum* IN THE DIAGNOSIS OF VISCERAL LARVA MIGRANS BY WESTERN BLOTTING TECHNIQUE

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SUMMARY

Visceral larva migrans (VLM) is a clinical syndrome caused by infection of man by *Toxocara* spp, the common roundworm of dogs and cats. Tissue migration of larval stages causes illness specially in children. Because larvae are difficult to detect in tissues, diagnosis is mostly based on serology.

After the introduction of the enzyme-linked immunosorbent assay (ELISA) using the larval excretory-secretory antigen of *T. canis* (TES), the diagnosis specificity was greatly improved although cross-reactivity with other helminths are still being reported. In Brazil, diagnosis is routinely made after absorption of serum samples with *Ascaris suum* antigens, a nematode antigenicaly related with *Ascaris lumbricoides* which is a common intestinal nematode of children.

In order to identify *T. canis* antigens that cross react to *A. suum* antigens we analyzed TES antigen by SDS-PAGE and Western blotting techniques. When we used serum samples from patients suspected of VLM and positive result by ELISA as well as a reference serum sample numerous bands were seen (molecular weight of 210-200 kDa, 116-97 kDa, 55-50 kDa and 35-29 kDa). Among these there is at least one band with molecular weight around 55-66 kDa that seem to be responsible for the cross-reactivity between *T. canis* and *A. suum* once it disappears when previous absorption of serum samples with *A. suum* antigens is performed.

KEYWORDS: *Toxocara canis* antigens; Immunodiagnosis; Visceral larva migrans; *Ascaris suum*; Western blotting.

INTRODUCTION

Visceral Larva Migrans (VLM) is a clinical syndrome of man caused by tissue migration of larval stages of *Toxocara canis*, the common roundworm of dogs and cats. Transmission to human beings occurs more frequently by ingestion of contaminated soil or of eggs on hands and fomites. Direct contact with infected dogs and cats plays a secondary role in transmission because eggs need an extrinsic period to become infective. Diagnosis of this disease depends mostly on immunological tests because neither eggs nor larvae are eliminated by the host[3].

Since the studies made by SAVIGNY[10] the antigens mostly used for the immunodiagnostic tests derive from larvae cultivated in vitro and are referred to as *Toxocara* excretory-secretory (TES) antigens[11].

Not all TES antigens are species or genus specific and serum samples from patients with ascariasis, filariasis and strongyloidiasis show reactivity with TES antigens by enzyme-linked immune assay (ELISA), immunoprecipitation and western blotting (WB)[1, 2, 7, 8, 9, 12].

In most areas in which *A. lumbricoides* is endemic, exposure to *A. suum* and *T. canis* is likely to be sufficiently common to confuse serodiagnosis[5]. Reactivity of serum to TES antigens is reduced by absorption with extracts of a wide variety of non homologous parasites[11]. In Brazil, diagnosis is routinely made by ELISA after absorption of serum samples with *Ascaris suum* antigens, a nematode antigenicaly related with *Ascaris lumbricoides* which is a common intestinal nematode of children[1].

This study aimed at evaluating cross-reactivity between *A. suum* and *T. canis* ES antigens by western blotting[13] technique using serum samples from patients with positive diagnosis to VLM, before and after absorption of these sera with *A. suum* antigens.

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MATERIAL AND METHODS

Toxocara canis excretory-secretory antigens (TES)

Female adults of *T. canis* were collected from feces of puppies treated with piperazine adipate (100 mg/kg). After collection worms were washed in saline and fertile eggs were obtained from the uteri of gravid female and left to embryonated for 30 days at 28° C in formalin solution. Secretory-excretory antigen was prepared by the method of SAVIGNY[10] with some modifications. Briefly, eggs were hatched mechanically to allow the migration of larvae in a Baerman apparatus and were maintained in Minimal Essential Medium until their viability fell significantly. Culture medium was collected every seven days, then concentrated by ultrafiltration (PM10, Amicon, Lexington, USA) and protein content was estimated by biciconinic acid kit-BCA (PIERCE).

Ascaris suum adult stage extract

Female worms were collected from swine intestine and washed in saline. Adult extract was prepared as described by CAMARGO et al.[2] and protein concentration was estimated by BCA kit (PIERCE).

Human sera

Two serum samples from healthy individuals and 4 serum samples from patients with clinical signs of VLM were evaluated. Sera from healthy individuals were negative and patients samples were positive by ELISA performed at Adolfo Lutz Institute as described by CAMARGO et al.[2] A positive reference serum sample was kindly provided by the Center for Disease Control (Georgia - USA).

Electrophoresis procedure

TES antigens were fractionated by polyacrylamide gel electrophoresis with dodecil sulfate (SDS-PAGE) according to LAEMMLI[6]. A 5-15% gradient running gel and a 3% acrylamide stacking gel was used. Either 500 µg or 300 µg of TES antigens were boiled with sample buffer (Tris/HCl 0.01M, pH 6.8; 1% SDS, 3% 2-mercaptoethanol, 0.1% bromophenol blue, 10% glycerol) for 1 min and applied to a 140 mm × 160 mm × 1 mm poliacrylamide gel. Sample was electrophoresed with constant voltage (50 V) until bromophenol blue had entered the gel when it was increased to 100 V until 0.5 cm from the edge of the gel. Electrode buffer, pH 8.3, contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Molecular weight standards (Sigma SDS-200) were included to calculate relative molecular weights.

Western blotting (WB) procedure

Transfer was performed according to TOWBIN et al.[13] in a TransBlot SD cell apparatus (Bio-Rad) using 15 V applied over 1.5 hours to a 0.45 µm nitrocellulose membrane in 0.025 M Tris/glycine buffer.

Nicrocellulose strips containing transferred proteins were rinsed with PBS and incubated for 2 hours with 5% skimmed milk to block remaining free sites and test sera diluted 1:320 were incubated for 1 ½ hour. Following 4 washes with PBS and 5% skimmed milk to remove unbound antibody, strips were incubated 1 hour in a horseradish peroxidase conjugated anti-human IgG (Sigma A-8792) diluted 1:3,000. After 4 washes with PBS and 5% skimmed milk substrate solution containing 0.0165% 3'3-'diaminobenzidine and 0.15% hydrogen peroxide was added and bands were visible within 5 min.

Tested sera were assayed before and after 1 hour absorption with *A. suum* antigen diluted 1:50 in PBS with 5% skimmed milk, at room temperature.

RESULT AND DISCUSSION

Figure 1 shows that pattern displayed by sera from patients with clinical signs of VLM is similar to that displayed by the positive reference serum (Fig. 2). Several bands can be seen and among these five major components can be identified: higher than 205 kDa, around 205 kDa, 116-97 kDa, 55-50 kDa and 35-29 kDa. Sera from healthy individual did not show any reactivity (data not shown). These data correspond to those reported in the literature.

MAIZELS et al.[9], in an analysis of radio-iodinated TES by SDS-PAGE found five major components and established two groups: low molecular weight (LMW) fractions (35 kDa) and high molecular weight (HMW) fractions (120 kDa). Applying

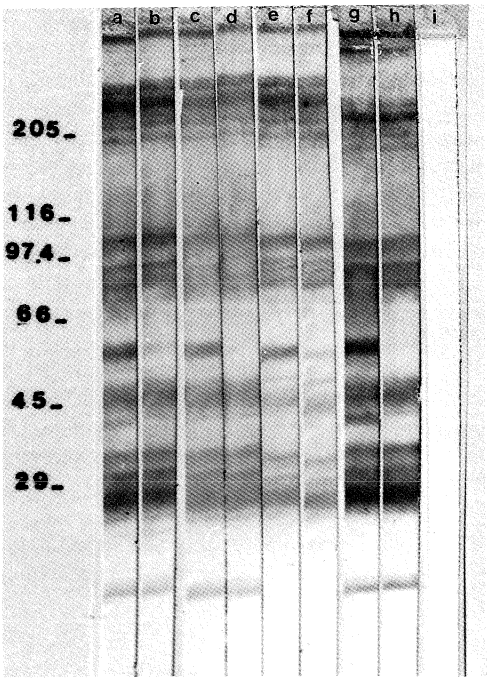

Fig. 1 – Western blotting of *Toxocara canis* ES antigen (500 µg). (a); (c); (e) and (g); patients sera 1/320; (b); (d); (f) and (h) same sera absorbed with *Ascaris suum* antigen 1/50; (i) conjugate control.

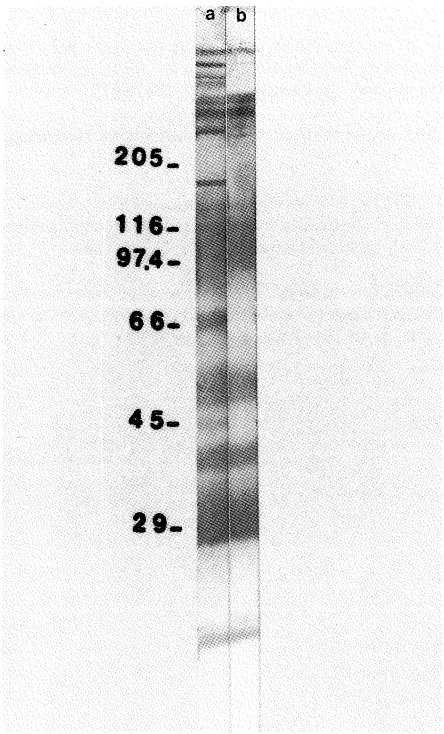

Fig. 2 – Western blotting of *Toxocara canis* ES antigen (300 µg). (a) positive control serum 1/320; (b) same serum absorbed with *Ascaris suum* antigen 1/50.

western blotting (WB) procedures to sera from VLM patients, MAGNAVAL et al.[8] found a typical pattern of 7 bands also divided into two groups: LMW fractions (24, 28, 30 and 35 kDa) and HMW fractions (132, 147 and 200 kDa). These authors suggested that LMW antigenic fractions might be more specific than HMW. In our study LMW fractions were also observed and did not show any cross-reactivity with *A. suum* antigens.

JACOB[4] studying the follow-up reactivity pattern of 12 serum samples by WB observed four bands with molecular weight between 29 and 210 kDa. Molecular band of 66 kDa was also observed in some patients sera as well as in some of the negative control sera. In our experiment similar band (55-66 kDa) was also observed from all patients sera. It seems to be responsible for cross-reactivity between *T. canis* and *A. suum* once it disappears when previous absorption of serum samples with *A. suum* antigens is performed. Besides these, positive reference serum shows 4 bands of high molecular weight (> 205 kDa) and another band around 200 kDa that also disappear after absorption.

LYNCH et al.[7] studying cross-reactivity of TES and other parasites recognized a band by WB with molecular weight of 81 kDa as the responsible for a strong cross-reactivity between *T. canis* and *Ascaris* extract. As suggested by SCHANTZ[11] reported differences in molecular weights of fractions can presumably reflect technical variations in detection and resolution of constituents or in methods for maintenance of larvae.

CONCLUSION

Cross-reactivity between *T. canis* and *A. suum* could be observed also by western blotting technique and this reinforce the necessity of absorption of serum samples from patients from tropical regions with extracts of other parasites as recommended by LYNCH et al.[7].

RESUMO

Reações cruzadas entre *Toxocara canis* e *Ascaris suum* no diagnóstico da Larva Migrans Visceral através da técnica de western blotting

A Larva Migrans Visceral (LMV), uma zoonose emergente, é uma síndrome clínica decorrente da infecção humana por larvas de *Toxocara* spp, parasita intestinal de cães e gatos. A dificuldade de detecção das larvas nos tecidos e a inespecificidade dos sinais clínicos tornam os testes sorológicos os meios diagnósticos mais adequados. Os antígenos excretores-secretores de *T. canis* (TES) empregados no teste ELISA embora tenham contribuído para melhorar a especificidade deste, apresentam reações cruzadas com diversos parasitas, particularmente com *Ascaris* sp. No Brasil o diagnóstico é rotineiramente feito após a absorção das amostras de soro com antígenos de *A. suum*, nematóide antigenicamente relacionado a *A. lumbricoides* o qual é comumente observado em crianças.

O presente trabalho objetivou evidenciar frações protéicas que apresentem reações cruzadas com antígenos de *Ascaris suum* através do western blotting. Soro de indivíduos sadios bem como soro de pacientes com suspeita clínica de LMV, além de uma amostra de soro controle positiva foram utilizadas na avaliação. Observou-se padrão de reatividade composto por frações de 210-200 kDa, 116-97 kDa, 55-50 kDa e 35-29 kDa.

Observou-se também reatividade dos soros a pelo menos uma proteína de 55-66 kDa responsável pela reatividade cruzada observada entre *T. canis* e *A. suum*.

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