

EVALUATION OF *DIROFILARIA IMMITIS* EXCRETORY/SECRETORY PRODUCTS FOR SEROEPIDEMIOLOGICAL STUDIES ON HUMAN DIROFILARIOSIS

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Summary :

The usefulness of excretory/secretory (E/S) products of adult *Dirofilaria immitis* for the diagnosis of human dirofilariosis was evaluated in 97 human sera using an ELISA and a Western Blot analysis with E/S antigens. Results of the ELISA analysis show that in healthy individuals from an endemic area seropositivity is high (22 %). Immuno Blot analysis shows that the reaction pattern with E/S antigens differentiates healthy seropositives, which recognize a 43 kDa band, from patients with pulmonary dirofilariosis, which specifically recognize bands between 22-28 kDa.

KEY WORDS : human dirofilariosis. *Dirofilaria immitis*. excretory/secretory antigens. serodiagnosis. ELISA. Western Blot.

Résumé : ÉVALUATION DES PRODUITS D'EXCRÉTION/SÉCRÉTION DE *DIROFILARIA IMMITIS* POUR L'ÉTUDE SÉROÉPIDÉMIOLOGIQUE DE LA FILARIOSE HUMAINE.

L'utilité des produits d'excrétion-sécrétion (E/S) de *Dirofilaria immitis* adultes dans le diagnostic de la dirofilariose humaine a été évaluée avec 97 sérums humains, en utilisant des antigènes E/S. Les résultats de l'analyse effectuée avec ELISA montrent un taux élevé de séropositivité (22 %) chez les individus sains d'une région endémique. L'analyse par Immuno-blot montre que le modèle de réaction recourant aux antigènes E/S permet de différencier les séropositifs, mettant en évidence une bande de 43 kDa, des patients avec dirofilariose pulmonaire, lesquels présentent spécifiquement des bandes entre 22 et 28 kDa.

MOTS CLÉS : dirofilariose humaine. *Dirofilaria immitis*. antigènes d'excrétion/sécrétion. diagnostique sérologique. ELISA. Western Blot.

INTRODUCTION

Dirofilaria immitis is the agent of a zoonosis widely distributed in canids of tropical and temperate areas. In previous studies we and others have shown that, in areas of canine endemia, asymptomatic human infections detected serologically are common, although solitary pulmonary nodules – the clinical hallmark of most described cases of human dirofilariosis – are rarely found (Konishi, 1989; Muro *et al.*, 1990; Villanueva and Rodríguez Pérez, 1993).

E/S products have shown their diagnostic usefulness in various helminthiosis such as fasciolosis (Hillyer and Soler de Galanes, 1988), toxocarosis (Savigny and Tizzard, 1977) and onchocercosis (Lobos *et al.*, 1991). In human dirofilariosis adult somatic antigens have been used both for epidemiological studies (Konishi, 1989; Muro *et al.*, 1990; Simón *et al.*, 1991; Villanueva and Rodríguez Pérez, 1993) and for diagnosis (Sato *et al.*, 1985; Glickman *et al.*, 1986; Cordero *et al.*, 1992 a, b). E/S products have been

used only once (Akao *et al.*, 1991) to study the pattern of antigenic recognition in Western Blot by a small number of sera from patients diagnosed as having pulmonary dirofilariosis.

This work was designed to investigate whether adult *Dirofilaria immitis* E/S products are a useful alternative source of antigens for the immunodiagnosis of human dirofilariosis.

MATERIALS, SUBJECTS AND METHODS

ANTIGENS

Dirofilaria immitis excretory/secretory antigens (DiE/S). Live worms were obtained from naturally infected dogs and washed in 0.01 M of phosphate buffer saline (PBS), pH 7.2 at room temperature. Twenty five worms were incubated for five days in 50 ml PBS at 37°C in Eagle Minimum Essential Medium (MEE) (Sigma Chemical Company, St. Louis, MO, USA), supplemented with gentamycin (0.04 %) and nistatin (0.01 %). The medium was changed daily. All fractions were dialyzed against 0.01M PBS, pH 7.2, and the suspension containing DiE/S antigens concentrated by filtration through an Amicon YC05 (Amicon Corporation Scientific Systems Division, Danvers, MA, USA). The

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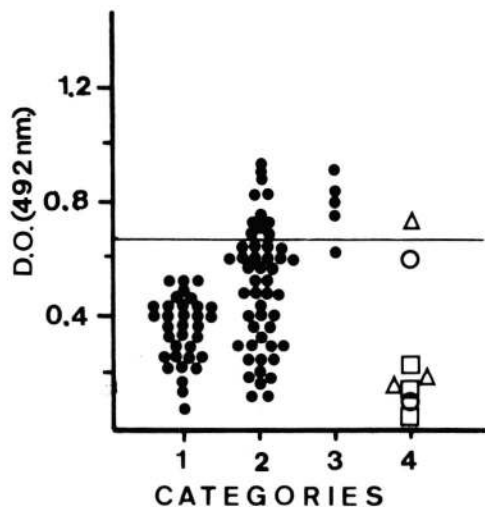


Fig. 1. – Results of the screening of 97 human sera by ELISA with excretory/secretory antigens of *Dirofilaria immitis*. Category 1 : 34 sera from healthy individuals residing in an area free of dirofilariosis. Category 2 : 50 sera from healthy individuals residing in an area with endemic dirofilariosis. Category 3 : 5 sera from patients diagnosed of pulmonary dirofilariosis. Category 4 : sera from patients diagnosed of : (O) hydatidosis; (Δ) pulmonary carcinoma; (\square) tuberculosis. Results are the mean of four replicates for each serum samples.

amount of proteins was measured by the Bradford method and adjusted to a final concentration of 1 mg/ml. The antigen extract was stored at -20°C until use.

PATIENTS

The following sets of human sera were analyzed : 1) Sera from individuals living in an area free of canine dirofilariosis (non-endemic sera). 2) Sera from individuals living in an area endemic for canine dirofilariosis without radiological pulmonary lesions (endemic sera). 3) Sera from patients diagnosed as having pulmonary dirofilariosis. 4) Sera from individuals with hydatidosis, pulmonary tuberculosis and pulmonary epidermoid carcinoma. All patients were studied at the Salamanca University Hospital.

IMMUNOLOGICAL TESTS

ELISA with E/S antigens was carried out as described by Simón *et al.* (1991) with minor modifications. The antigen concentration in the assays was 4 $\mu\text{g}/\text{ml}$. All sera were tested at 1:100 dilution. Anti-human IgG conjugated to horseradish peroxidase (Dako, Denmark) was used at 1:4000 dilution. The optical density (OD) was measured in an Easy-Reader EAR 400 FT at 492 nm (SLT Labinstrument, Austria).

Western Blot (WB) was carried out as described by Tsang *et al.* (1985). Proteins were separated on 8-

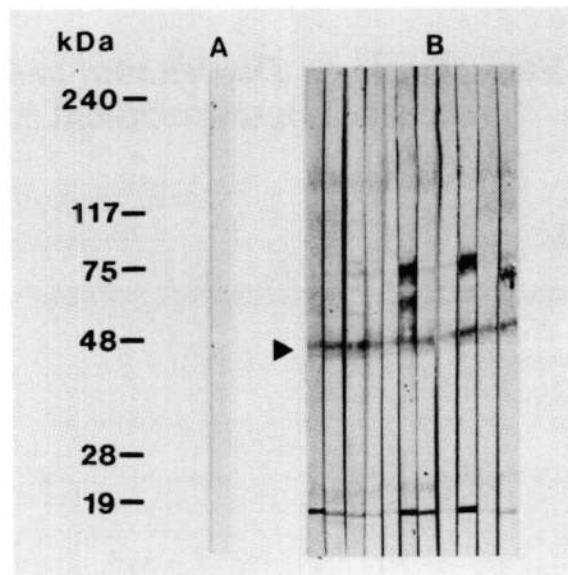


Fig. 2. – Immuno Blot analysis with excretory/secretory antigens of the 11 sera from healthy individuals residing in an area of endemic dirofilariosis, positive to ELISA E/S (B); blank (PBS) (A). Arrowhead points to the 43 kDa polypeptide recognized by these sera.

20 % gradient gel slabs in accordance to the method of Laemmli (1970) in a Miniprotean II (Bio-Rad Laboratories, Inc.USA). All samples were treated with 0.15 % dithiothreitol, 2 % SDS, 1M Tris-HCl (pH 6.8), 10 % glycerol and 0.2 % bromophenol blue. Samples were heated to 100°C for 3 minutes in a water bath and then transferred to nitrocellulose. Human sera were used at a 1:40 dilution. Anti-human IgG-peroxidase conjugate (Dako, Denmark) was employed at a 1:500 dilution.

STATISTICAL ANALYSIS

Student's T test was used to detect differences between groups.

RESULTS

ELISA ANALYSIS OF HUMAN SERA

97 sera from the different categories were studied by ELISA. Results are shown in Figure 1.

ELISA positivity limit was defined as 3 standard deviations above the mean of the optical density (OD) of 34 non endemic sera; thus positivity was defined as an OD greater than 0.65. The OD of 50 endemic sera ranged from 0.190 to 0.929; 11 of them surpassed the limit of positivity. Four of the five sera from indivi-

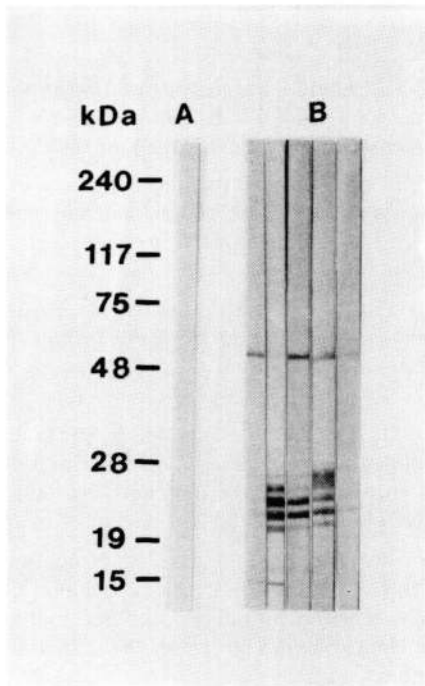


Fig. 3. – Immuno-Blot analysis of 5 sera from patients diagnosed of pulmonary dirofilariosis (B); blank (PBS) (A).

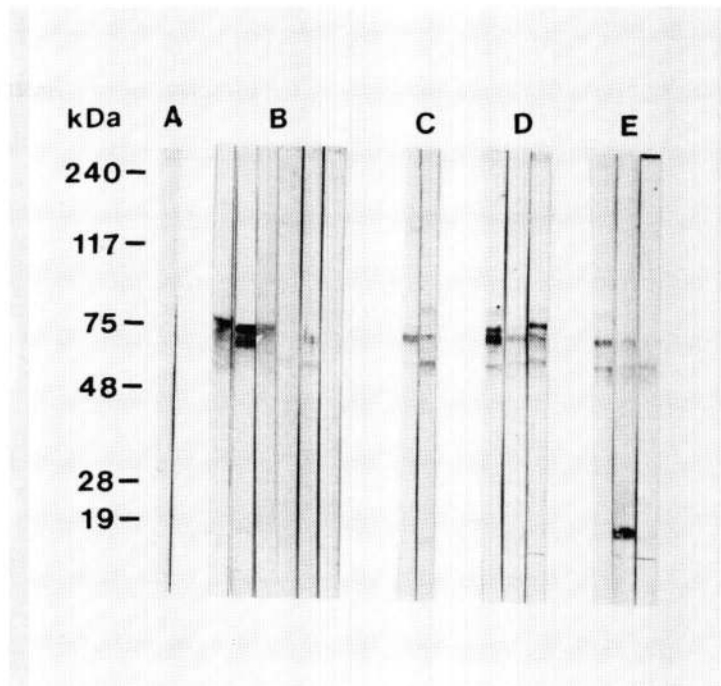


Fig. 4. – Immuno Blot analysis of sera from: Healthy individuals from a non endemic area (B); patients with hydatidosis (C); patients with pulmonary carcinoma (D); patients of tuberculosis (E); blank (PBS) (A).

duals diagnosed as having pulmonary dirofilariosis were positive, with values of 0.78; 0.8; 0.82 and 0.92; the fifth had an OD of 0.58. Only one serum from a patient diagnosed of pulmonary epidermoid carcinoma was positive, with an OD of 0.72; all other sera from individuals with diseases other than dirofilariosis had OD's below the positivity limit, ranging between 0.64 and 0.04, with a mean of 0.25. Statistical analysis shows that there are differences between the non endemic sera and endemic sera without pulmonary alterations ($p=0.00027$), between the endemic sera without pulmonary alterations and the sera of dirofilariosis patients ($p=0.0019$), and between endemic sera without pulmonary alterations and sera of patients with pulmonary diseases other than dirofilariosis ($p=0.0313$). No differences were found between the OD of the non endemic sera and the sera of patients with pulmonary diseases other than dirofilariosis.

WESTERN BLOT ANALYSIS OF HUMAN SERA

Endemic sera that were seropositive in the ELISA strongly recognized a 43 kDa band in WB (Fig. 2). Other bands of >117, 21 and 18 kDa are also recognized by these sera, although less intensely. Some sera, moreover, also recognize bands between 50 and 75 kDa.

Analysis of sera from patients diagnosed as having pulmonary dirofilariosis (Fig. 3) shows a different pattern of antigen recognition. All sera recognized bands

between 21-28 kDa; four of them also recognized a band with MW >48 kDa, and two of them a band of 15 kDa. Figure 4 shows the pattern of antigen recognition in E/S products by negative non endemic sera in ELISA and by sera from patients with other diseases. Only bands in the molecular range of 58-75 kDa are recognized; the serum of a patient with pulmonary tuberculosis also binds a band of 18 kDa; the serum of the patient with pulmonary epidermoid carcinoma which resulted seropositive by ELISA showed the same recognition pattern.

DISCUSSION

Our results show that the ELISA to detect antibodies against E/S antigens from *D. immitis* can clearly differentiate between the four sets of sera analyzed. On the other hand, E/S antigens are able to detect a great number of individuals with infection by the parasite but without pulmonary lesions. The seroprevalence in healthy individuals from the endemic area was 22 %; we have previously found a seroprevalence of 5.8 % in the same area using an ELISA with adult somatic antigens (Simón *et al.*, 1991). The different seroprevalence with these two antigenic sets suggests that the ELISA with E/S antigens is more sensitive in the detection of contact with the parasite, and therefore, more useful for seroepidemiological studies.

Moreover, Immuno-Blot analysis detects important differences in the pattern of antigen recognition by the four sets of sera. These differences are clearest in the recognition of a band of apparent MW of 43 kDa intensely and exclusively bound by all endemic sera positive to ELISA E/S. A group of bands between 20-28 kDa, on the other hand, is recognized only by the sera of patients with pulmonary dirofilariasis. Other specificities are of less interest, either because they are due probably to non specific reactivity (75-50 and 18 kDa), or because both categories of sera recognized them (20 kDa). Additionally, Immuno-Blot is able to resolve the non specific ELISA E/S reactivity of the sera from a patient with pulmonary carcinoma; the pattern this serum shows in Immuno-Blot is similar to that of the negative endemic and to that of the sera or from other diseases, not recognizing either the 43 kDa or 20-28 kDa antigens.

The fact that only healthy individuals react with this 43 kDa antigen could be compared with a similar fact found in bancroftian filariasis, in which non-infected individuals living in an endemic area also recognize strongly a 43 kDa larval antigen; as in this case, it can be speculated that this protein confers, or is a marker of, protective immunity in exposed people from endemic areas (Freedman *et al.*, 1989).

The pattern of protein recognition in WB by the sera of patients with pulmonary dirofilariasis is different. Akao *et al.* (1991) examined seven such sera by WB with E/S antigens. Six of them recognized bands with apparent MW of 123-83, 60-47, 20-19.5, 18, 17.5-17, and 14 kDa; a protein of 18 kDa is recognized by all analyzed sera, including a negative control. In a previous work we have described a 22 kDa protein in the somatic antigen complex of adult *D. immitis* recognized by the sera of three patients with pulmonary dirofilariasis but not by seropositive endemic sera (Perera *et al.*, 1994). The results of the present study are consistent with this finding, because in the cluster of bands from the E/S antigenic complex between 20-28 kDa a 22 kDa band is present.

To conclude, ELISA to detect IgG antibodies against *D. immitis* E/S proteins seems to be more sensitive in the detection of contact with the parasite than the same test using somatic antigens. By Immuno-Blot analysis, a 43 kDa protein is detected which could serve as a marker of healthy individuals with prior contact with the parasite; its possible protective role can only be speculated. Lastly, our study with E/S proteins confirms our previous data that a 22 kDa protein of adult *Dirofilaria immitis* is a useful marker to discriminate patients with pulmonary dirofilariasis from healthy seropositives.

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