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Short communication

Identification of *Dirofilaria immitis* immunoreactive proteins recognized by sera from infected cats using two-dimensional electrophoresis and mass spectrometry

Javier González-Miguel[a], Rodrigo Morchón[a], Mar Siles-Lucas[b], Ana Oleaga[b], Fernando Simón[a,*]

[a] *Laboratory of Parasitology, Faculty of Pharmacy, University of Salamanca, Av. Campo Charro s/n, 37007 Salamanca, Spain*
[b] *Laboratory of Parasitology, Instituto de Recursos Naturales y Agrobiología de Salamanca (CSIC), Cordel de Merinas, 40-52, 37008 Salamanca, Spain*

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ABSTRACT

The aim of the present work was to identify proteins of *Dirofilaria immitis* recognized by the immune system of naturally and experimentally infected cats, using two-dimensional electrophoresis and mass spectrometry. Thirty-five immunoreactive proteins of *D. immitis* were identified. These proteins are involved in metabolism, plasminogen binding, anti-oxidant and detoxificant activity, up-regulation of the Th2 anti-inflammatory response and other processes. The timing evolution of this recognition pattern indicated that at 2 months post-infection a wide recognition of many parasite proteins belonging to many functional groups is still observed, increasing progressively during the course of the infection. The real effect on the vital capacity of *D. immitis* worms and on the development of pathological events of feline dirofilariosis will be investigated in the future.

Cardiopulmonary dirofilariosis is a vector-borne parasitic disease affecting primarily dogs and cats worldwide. Moreover human infections are found in endemic areas because some mosquito species transmitting dirofilariae can also feed on humans [1,2]. The development of *Dirofilaria immitis* and subsequent pathological effects are different in each host. In cats, infections are produced by a very limited number of worms that reach maturity after a longer period than in dogs. Their lifespan is shorter than in canine host and microfilariae are absent [3]. Feline dirofilariosis has not an univocal clinical course and is typically acute and elusive [4]. In spite of the worms located in pulmonary arteries cause local arteritis, some cats never manifest clinical symptoms. When these appear they are associated with the arrival and death of both immature and mature worms into the pulmonary arteries [5]. Vascular and parenchimal inflammation, thromboembolisms, pulmonary infarct and hemorrhages with circulatory collapse and respiratory failure have been described [6].

Information on the pathogenic and survival mechanisms of *D. immitis* in feline host is more limited. Prostaglandin E_2 (PGE_2), related to an anti-inflammatory response, predominates during the first two months of the feline infections, switching to a predominance of thromboxane B2 (TxB2) (pro-inflammatory) accompanied with high levels of anti-*Wolbachia* surface protein (WSP) antibodies 6 months post-infection (pi), concurrent to the arrival of adult worms to the pulmonary arteries [7,8]. On the other hand, several immunogens have been previously identified in canine dirofilariosis by proteomic techniques [9]. The goal of the present study is the identification of immunoreactive proteins from *D. immitis* potentially related with parasite pathogenic and survival mechanisms in cats.

Serum samples from 11 cats with naturally acquired cardiopulmonary dirofilariosis and 10 healthy cats were used. Parasitological status of infected cats was tested by ELISA to detect antibodies against *D. immitis* and *Wolbachia* [7] and by echocardiography to visualize adult worms. Additionally, symptoms were also assessed. Only cats with positive results in the three above-mentioned cases were included in the study. Sera from 8 experimentally infected cats collected at 0, 2, 4 and 6 months pi were also used.

Parasite extract DiSB, preparation of samples, two-dimensional electrophoresis (2-DE), immunoblots and mass spectrometry (MS) were performed as described before [9] with minor modifications. Immunoblots were carried out with a pool of 10 sera from healthy cats, a pool of 11 sera from naturally infected cats and 4 pools of 8

Abbreviations: pi, post-infection; PGE_2, prostaglandin E_2; TxB2, thromboxane B2; WSP, *Wolbachia* surface protein; 2-DE, two-dimensional electrophoresis; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; DiSB, soluble extract of proteins from *Dirofilaria immitis* adult worms; Ig, immunoglobulin; pI, isoelectric point; MW, molecular weight; HSP, heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

* Corresponding author. Tel.: +34 923 294535, fax: +34 923 294515.
E-mail address: fersimon@usal.es (F. Simón).

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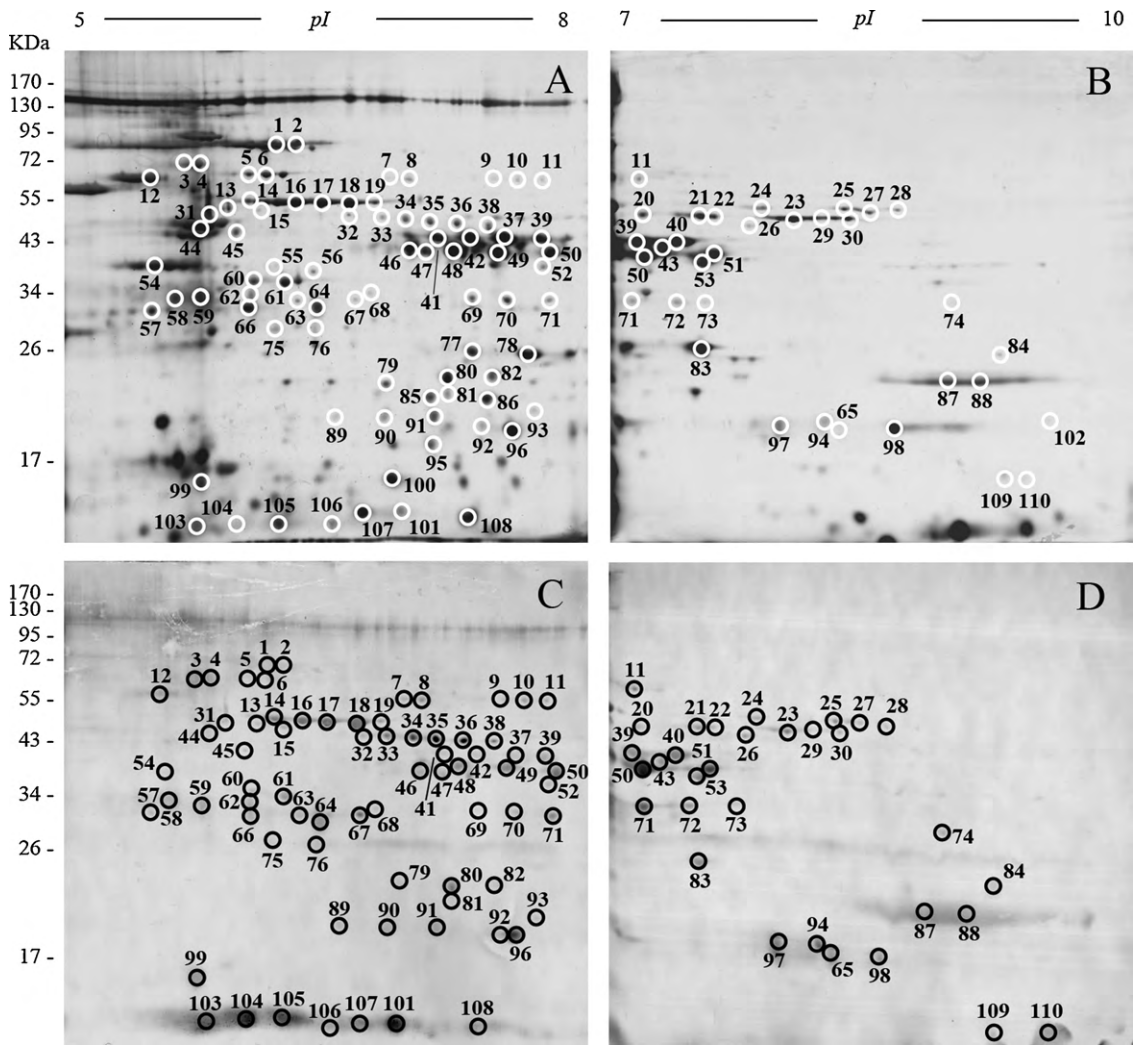

Fig. 1. Representative two-dimensional electrophoresis of 40 μg of the DiSB extract from adult *D. immitis* worms (A and B). The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained, and two-dimensional Western blot showing the antigenic spots of the DiSB extract (C and D) revealed by pools of serum samples from naturally infected cats. Reference molecular masses are indicated on the left. The antigenic spots analyzed by mass spectrometry are circled and numbered.

sera each from experimentally infected cats collected at different time points pi (above-mentioned), at 1:400 dilution. A horseradish peroxidase-labelled anti cat IgG (Fitzgerald) at 1:2000 dilution was also used. Samples were analyzed in triplicate to assess the overall reproducibility of the protein and immunogen spot patterns. Finally, for MS analysis, the spots containing immunogenic proteins were excised manually from the gels and sent to the Unit of Proteomics of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, Spain).

The 2-D gels of DiSB proteins showed 489 spots over a broad range of MW (10–170 kDa), 401 of them with p*I*s between 5 and 8 and 88 with p*I*s between 8 and 9.8 (Fig. 1A and B). Immunoblot analysis of the DiSB carried out with serum samples from naturally infected cats revealed 105 major antigenic spots (Fig. 1C and D). This represents a recognition rate of 21.47% over the total of protein spots. The matching of spots detected by western blotting with their homologous in the silver nitrate stained 2-D gels allowed us to select a total of 101 antigenic spots of *D. immitis.*

The timing evolution of the recognition pattern during the course of infection was studied using serum samples from experimentally infected cats at 2, 4 and 6 months pi. These sera recognized 50, 86 and 116 major antigenic spots, respectively (Fig. 2), corresponding to recognition rates of 10.22, 17.59 and 23.72%. These represent successive recognized spots, since those found at 2 moths pi were also found at 4 and 6 months pi, and those recognized by 4 months pi sera were also recognized by cats after 6 months of infection. Analysis of the 2-D images allowed the localization of 48, 84 and 110 spots, respectively. Up to 101 of the 110 spots recognized by sera from experimentally infected cats at 6 months pi were also recognized by serum samples from naturally infected cats. Nine spots (numbers 55, 56, 77, 78, 85, 86, 95, 100 and 102) were recognized by sera from 6 months pi experimentally infected cats (Fig. 2), but not from naturally infected cats (Fig. 1). In addition, spots 78, 86 and 100 were also recognized by sera from 4 months pi experimentally infected cats.

Immunogenic spots developed by sera from naturally and experimentally infected cats showed a wide range of apparent MW and p*I*s (between 10 and 125 kDa, and 5.1 and 9.2, respectively). Serum samples from healthy animals (negative control) and from experimentally infected cats before infection did not recognize any DiSB protein in 2-D gels (data not shown). Only minor differences were observed among both gel or immunoblot triplicates.

The antigenic spots recognized by naturally infected cats ($n = 101$) and additionally those specifically recognized by experimentally infected cats at 6 months pi or at 4 and 6 months pi ($n = 9$) were manually excised from 2-D gels and submitted to analysis by MS. Up to 76 of the 110 analyzed spots (69.1%) were identified, representing 35 different proteins.