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

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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.

Article

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 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.

Table 1 shows the identity of these proteins, their theoretical MW and p*I*s, the access number to the homologous protein available in the NCBI database, the sequence coverage, the Mascot score and the biological process in which the proteins identified are involved according to the gene ontology database (http://www.geneontology.org) and the Swiss-Prot/UniProt database (http://beta.uniprot.org).

The aim of the present work is to identify immunoreactive proteins recognized by the immune system of cats infected with *D. immitis*, within the context of parasite/host relationships in the feline dirofilariosis. Many of the identified proteins are related to cellular metabolism. Seven of these are glycolytic enzymes and three are related to other energy generation and metabolic pathways (see Table 1). *D. immitis* adult worms and other filarial species can be considered homolactic fermenters because the lactate produced from the piruvate is the main or unique final product of the carbohydrate catabolism [10]. On the other hand, it has been indicated that malate produced in the Krebs cycle can revert to piruvate by the activity of malic enzyme [10]. Some of these enzymes can develop other functions. For example, GAPDH and enolase bind plasminogen as it has been demonstrated in other organisms [11]. The activation of the fibrinolytic system in which plasminogen plays a key role, could be of special relevance in *D. immitis* adult worms that live in the host circulatory system for long periods of time. The production of specific antibodies against different plasminogen binding proteins by the immune system of cats could interfere their activity favoring the development of thromboembolisms, one of the most important pathological processes in feline dirofilariosis [12]. This is consistent with the high concentration of TxB2, responsible of the stimulus of inflammatory and obstructive reactions, previously detected in naturally infected cats and in cats experimentally infected at 6 months pi [8].

Infected cats recognize some proteins of *D. immitis* with anti-oxidant and detoxificant activity. The key cells in the immune response against filariae are monocytes and neutrophils [13], able to generate oxidative products derived from O_2 and NO. Thus, the anti-oxidant capacity of the filariae is fundamental for the long-term survival in immunocompetent hosts. The results obtained in the present work suggest that cats develop an antibody response against some of these molecules, fact that could have a detrimental effect in the survival of the worms.

Two isoforms of the HSP-70 and three small heat shock proteins (see Table 1) have been also identified in this study. The immunoreactivity of the HSP-70 has been observed previously in lymphatic filariasis and onchocerciasis [14], [15], as well as in other helminthic infections [16]. These proteins play important roles related to cellular survival in period of stress [17]. Moreover they can be expressed in normal conditions of growth and cellular division, acting as chaperone and control the correct folding and proteic transport [18].

Galectin-1 is a potent modulator of the immune response, able to down-regulate the Th1-type response and up-regulate the Th2-type response [19]. Recently, it has been observed that in humans exposed to *D. immitis*, the response of IgE antibodies (Th2 anti-inflammatory response) is stimulated, mainly, by galectin and aldolase-like molecules of the parasite [20]. Other studies have related the galectins with endothelial proliferation [21], the regulation of the alternative activation of macrophages [22] and the inhibition of the lymphocyte traffic [23].

The immunomic map of the experimentally infected cats after 4 months pi is very similar to that obtained in naturally infected cats, indicating that the two types of infections develop in a similar way and they are comparable. The immune system of the cat recognizes proteins of *D. immitis* related to many of the functional groups described after 2 months pi, increasing progressively the number of proteins recognized during the course of the infection. At 6 months pi, a phase in which worm maturation and location in the pulmonary arteries as well as the death

of some of them habitually occur, there exist a production of specific antibodies against a great number of parasite proteins, with very different functions.

To conclude, in the present work 35 immunoreactive proteins of *D. immitis* recognized by serum samples from infected cats have been identified. Many of these proteins could participate in key processes for the survival of the parasite like energy generation, plasminogen binding, anti-oxidant and detoxificant activity and the up-regulation of the Th2 anti-inflammatory response. Their recognition occurs before the maturation and location of adult worms into the pulmonary arteries. We do not know if this recognition has a real effect on the reduction of the vital capacity of *D. immitis* worms that can be related to the shortening of their life cycle in cats and to the death of some immature worms (4–6 months pi), key facts for the development of pathological events of feline dirofilariosis [11]. Future studies must be focused in the resolution of this and other fundamental questions in the parasite/host relationships in feline dirofilariosis.

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Figures

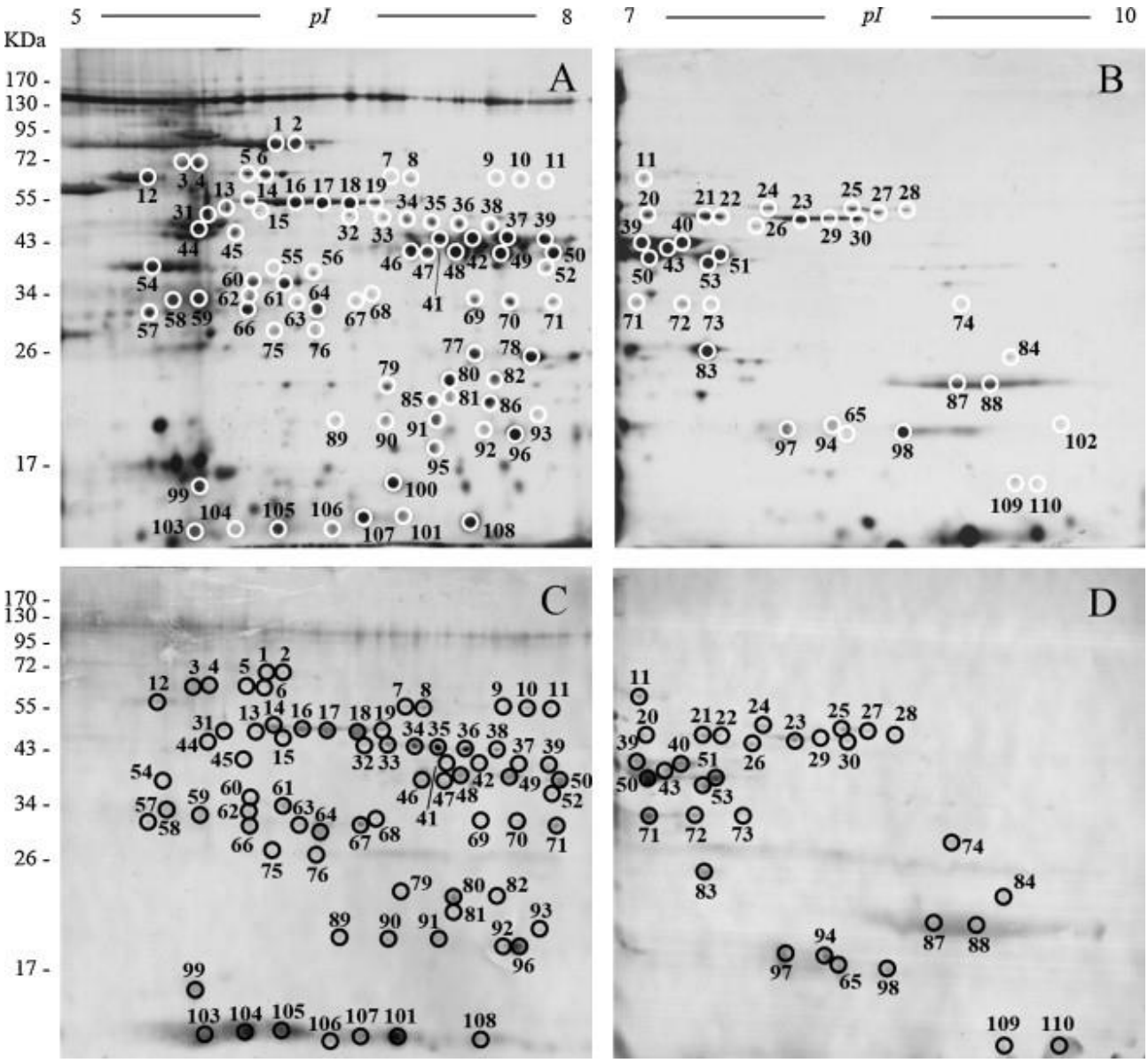

Fig. 1. Representative two-dimensional electrophoresis of 40 µg of the DiSB extract from adult *D. immitis* worms (A and B). The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained, and two-dimensional Western blot showing the antigenic spots of the DiSB extract (C and D) revealed 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.

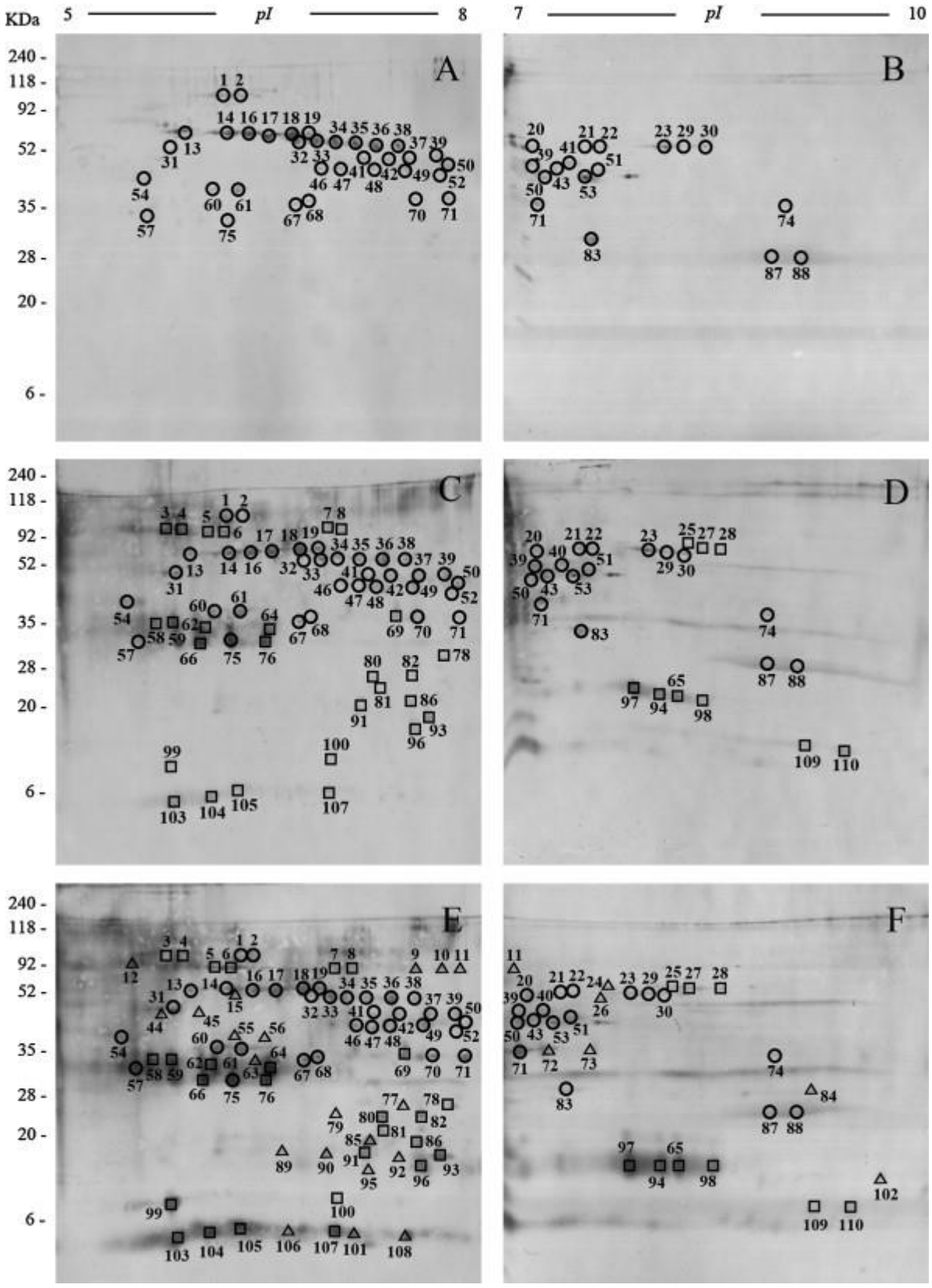

Fig. 2. Western blot showing the antigenic spots of the DiSB extract revealed by pools of serum samples from experimentally infected cats collected at 2 (A and B), 4 (C and D) and 6 (E and F) months pi. Reference molecular masses are indicated on the left. The antigenic spots analyzed by mass spectrometry are numbered and marked with a circle (2 months pi), square (4 months pi) or triangle (6 months pi).

Tables

Table 1. Antigenic protein spots from DiSB extract recognized by naturally and experimentally infected cats and identified by MALDI-TOF MS. MW (apparent molecular weight in kilodaltons (kDa)) and p*I* (isoelectric point) show the corresponding experimental values for each spot (for one and two spots) or the corresponding range values (for more than two spots). Sequence coverage (%) shows the percentage of sequence coverage for each identified spot. Mascot score is the score given as $S = -10 \times \log(P)$, where P is the probability that the observed match would be a random event. Mascot score values above 80 are considered significant ($P < 0.05$). Biological process in which the antigenic proteins of DiSB extract are involved was assigned according to the Gen Ontology and Swiss-Prot/Uniprot databases. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GP, glutathione peroxidase; PE, phosphatidyl-ethanolamine; Di-NCF, *Dirofilaria immitis* neutrophil chemotactic factor. (*) Spots differentially recognized by sera from experimentally infected cats and not from naturally infected cats.

Spot number	Accession code	Protein name	Species	MW (kDa)	p*I*	Sequence coverage (%)	Mascot score	Biological Process
1/2	AAF32254	Heat shock protein 70	*Wuchereria bancrofti*	79.8/79.8	6.1/6.3	12/19	204/304	Stress response
3/4	CAA34719	Actin	*Caenorhabditis elegans*	65.6/65.6	5.8/5.9	25/20	240/202	Cell motility
5/6	AAC24752	Transglutaminase precursor	*Dirofilaria immitis*	63.6/63.4	6.1/6.2	21/8	115/109	Redox homeostasis
7/8	AAV33247	Phosphoglycerate mutase	*Onchocerca volvulus*	61.1/61.6	7.0/7.1	9/6	158/152	Glycolysis
9–11	XP_001892448	FAD-dependent oxidoreductase	*Brugia malayi*	61.5–65.5	7.2–7.7	10/6/4	144/123/125	Electron transport
16–19	XP_001896281	Enolase	*Brugia malayi*	53.1–53.5	6.4–6.9	30/26/24/21	427/178/366/115	Glycolysis
20/21/23	XP_001891892	Phosphoglycerate kinase	*Brugia malayi*	54.7–54.8	7.3–8	4/4/13	104/149/139	Glycolysis
24–26	XP_001900957	Fumarase	*Brugia malayi*	53.7–57.7	7.7–8.3	2/3/2	91/106/100	Aerobic metabolism
31	EDP37909	Rab GDP dissociation inhibitor α	*Brugia malayi*	56.4	6.0	37	596	Protein transport
34–36	XP_001670614	Hypothetical protein CBG05397	*Caenorhabditis briggsae*	47.8–48.5	6.9–7.4	7/7/7	118/140/85	–
37	XP_001894530	Fructose-bisphosphate aldolase 1	*Brugia malayi*	44.2	7.6	12	124	Glycolysis
38–43	AAB52600	Fructose-bisphosphate aldolase	*Onchocerca volvulus*	43.9–52.0	7.2–7.8	15/9/28/20/18/15	128/112/248/178/185/263	Glycolysis
44	NP_505818	Actin 2	*Caenorhabditis elegans*	47.5	5.9	28	245	Cell motility
48/50	XP_001899850	GAPDH	*Brugia malayi*	42.0/41.5	7.9/7.3	21/20	349/311	Glycolysis
49/51	ACC77974	GAPDH	*Wuchereria bancrofti*	41.5/49.8	7.6/7.6	25/9	301/100	Glycolysis
52	XP_001900208	Lactate dehydrogenase	*Brugia malayi*	38.9	7.8	5	113	Anaerobic glycolysis
53	XP_001897743	Aldo/keto oxidoreductase	*Brugia malayi*	48.4	7.5	6	105	Redox process

54	XP_001899521	Disorganized muscle protein 1	*Brugia malayi*	39.1	5.6	17	143	Cell adhesion
55*	AAZ42332	G protein β subunit	*Caenorhabditis remanei*	37.9	6.2	7	134	Signal transduction
59/63–65	Q27384	Pepsin inhibitor Dit33 precursor	*Dirofilaria immitis*	22.7–34.0	5.9–8.3	26/26/20/21	201/194/183/178	–
66	NP_508842	Actin family member (act-4)	*Caenorhabditis elegans*	32.6	6.1	11	180	Cell motility
69	AAF37720	Galectin	*Dirofilaria immitis*	33.8	6.7	21	132	Immune response
70–72	XP_001900812	Galectin	*Brugia malayi*	33.7–34.0	7.4–7.8	11/14/15	116/203/172	Immune response
73	AAF63406	Galectin	*Haemonchus contortus*	43.4	7.4	9	133	Immune response
75	AAB08736	Small heat shock protein p27	*Dirofilaria immitis*	29.5	6.2	23	203	Stress response
76	P52033	GP, Di29 precursor	*Dirofilaria immitis*	29.6	6.5	17	121	Oxidative stress response
80/81	AAC38831	Thioredoxin peroxidase	*Dirofilaria immitis*	24.3/22.8	7.3/7.3	18/18	124/118	Redox homeostasis
83	XP_001897269	Triosephosphate isomerase	*Brugia malayi*	37.0	7.5	40	448	Glycolysis
84	CAA73325	Glutathione transferase	*Brugia malayi*	35.7	9.3	11	99	Detoxification
86*	AA799423	Peptidylprolyl isomerase	*Taenia solium*	22.6	7.6	13	119	Protein folding
87/88	AAD11968	P22U	*Dirofilaria immitis*	31.4/31.6	9.0/9.2	66/55	510/582	–
89–92	CAA48632	OV25-1 protein	*Onchocerca volvulus*	20.3–21.0	6.6–7.5	18/22/28/12	152/156/167/161	Stress response
93/94	BAA96354	PE-binding protein	*Dirofilaria immitis*	21.5/24.7	7.7/8.2	16/26	129/230	Signal transduction
96	XP_001899662	OV-16 antigen precursor	*Brugia malayi*	20.0	7.6	16	97	Signal transduction
97/98	AAC47233	Cyclophilin Ovcyp-2	*Onchocerca volvulus*	24.1/22.7	7.9/8.6	8/29	118/171	Protein folding
100*/101	XP_001895416	Small heat shock protein	*Brugia malayi*	16.1/14.9	6.9/6.9	12/8	155/86	Stress response
102*	XP_001902628	Bmcyp-2	*Brugia malayi*	18.1	9.6	25	144	Protein folding
105/107	BAA02004	Di-NCF precursor	*Dirofilaria immitis*	14.4/14.9	6.2/6.7	10/12	124/124	–
109/110	XP_001901495	Nucleoside diphosphate kinase	*Brugia malayi*	15.1/14.6	9.3/9.4	12/12	136/139	Nucleotide metabolism