

Galectin and aldolase-like molecules are responsible for the specific IgE response in humans exposed to *Dirofilaria immitis*

C. POU-BARRETO,¹ M. A. QUISPE-RICALDE,¹ R. MORCHÓN,² C. VÁZQUEZ,³ M. GENCHI,⁴ I. POSTIGO,⁵ B. VALLADARES¹ & F. SIMÓN²

¹Departamento de Parasitología, Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, Tenerife, Spain, ²Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain, ³Laboratorio de Análisis Clínico, Sección de Alergia e Inmunología, Hospital Universitario Ntra. Sra. de La Candelaria, Tenerife, Spain, ⁴Dipartimento di Patologia Animale, Igiene e Sanità Pubblica Veterinaria, Sezione di Patologia Generale e Parassitologia, Università di Milano, Milano, Italy, and ⁵Departamento de Inmunología, Microbiología y Parasitología, Facultad de Farmacia, Universidad del País Vasco, Vitoria, Spain.

SUMMARY

Dirofilaria immitis is the agent of the heartworm disease in canids and felids, and of pulmonary dirofilariasis in man. Like other filariae, *D. immitis* harbours endosymbiont *Wolbachia* bacteriae. In this work we analyse the response of specific IgE antibodies against both *D. immitis* antigens and the *Wolbachia* surface protein (WSP) in two groups of persons living in an area of canine endemia, one presenting high levels of total IgE (group 1) and other with normal levels (group 2). Infections with *D. immitis* were demonstrated by the presence of specific IgG in 228 individuals (48.8%) of the group 1 and only in one of the group 2. Specific IgE antibody response against *D. immitis* antigens was detected only in individuals of the group 1. IgE response against WSP was not detected in any group. The IgE response was directed mainly against two molecules of 33 and 42 kDa of the antigenic extract of *D. immitis*. These molecules were identified by mass spectrometry as a galectin and an aldolase, respectively. Their possible role in the survival mechanisms of the parasite and their contribution to development of allergic reactions in individuals resident in areas with heartworm disease are discussed.

Keywords Aldolase, atopic, *Dirofilaria immitis*, galectin, humans, IgE

INTRODUCTION

Dirofilaria immitis is the causal agent of canine and feline cardiopulmonary dirofilariasis in temperate and tropical areas all over the world (1). Because of culicid mosquitoes feed indistinctly on animal reservoirs and human hosts are present in many endemic areas, *D. immitis* is transmitted to man causing pulmonary dirofilariasis (2). This pathological condition is characterized by the development of benign pulmonary nodules in small branches of the pulmonary artery, as a consequence of the inflammatory reaction against the immature worms trapped in this location (3). The retrospective review of the literature indicates that the cases of pulmonary dirofilariasis appear almost frequently or they pass undetected. Nevertheless, seroepidemiological studies show high seroprevalences of anti-*D. immitis* antibodies in humans resident in endemic areas (4,5), indicating an intense risk of infection in areas where canine dirofilariasis exists (6,7). *D. immitis* harbours endosymbiont bacteria of the genus *Wolbachia* (8), which play an important role in the stimulus of the immune and inflammatory response during human and animal dirofilariasis (9,10).

Mechanisms developed by the helminth parasites to long-term survival in immunocompetent hosts are varied and complex. They include, among others, the up-regulation of the anti-inflammatory Th2-type response that promotes the down-regulation of the pro-inflammatory Th1-type response (11). The Th2-type response is characterized by the production of antibodies, whereas the Th1-type response is responsible of some immunopathologic mechanisms. Different parasitic molecules have been implicated in the modulation of the immune and allergic response of hosts infected by helminths. Among these molecules, the galectins and aldolases have demonstrated the ability to stimulate IgE antibody synthesis during the nematode infections (11,12). *D. immitis* stimulates a dual Th1/Th2-type response in infected dogs. The Th1-type response is stimulated by molecules of the endosymbiont

Correspondence: Fernando Simón, Laboratorio de Parasitología, Facultad de Farmacia, Universidad de Salamanca, Av. Campo Charro s/n, 37007 Salamanca, Spain (e-mail: fersimon@usal.es).

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Wolbachia bacteria, like the *Wolbachia* surface protein (WSP), while the Th2-type response is stimulated by *D. immitis* antigens (13,14). In human dirofilariasis, patients diagnosed as having pulmonary dirofilariasis produce IgG or IgM antibody response, but never synthesize IgE, while asymptomatic exposed individuals express a dominant response of IgE (5,15). Several studies have shown high levels of specific IgE antibodies against different species of filariae in allergic individuals with asthma or tropical pulmonary eosinophilia (16–18).

In the present work the IgE antibody response against *D. immitis* is analysed in atopic individuals resident in an endemic zone of canine dirofilariasis and the proteins implicated in the stimulus of these antibodies are identified and partially characterized.

MATERIALS AND METHODS

Serum samples

A total of 467 serum samples from humans with high levels of total IgE (group 1 = G1) and 83 with normal levels of total IgE (group 2 = G2) living in an endemic area of canine dirofilariasis (Tenerife, Canary Islands, Spain) (19) were employed. The levels of total IgE in blood were analysed previously by Modular Analytics E170 (Roche Diagnostics, Germany) with the IgE II Immunoglobulin E kit (Roche Diagnostics, Germany) at the service of Allergy and Immunology of the University Hospital Ntra. Sra. de Candelaria. Normal levels of total IgE were considered between 15 and 200 IU/mL depending of the age.

Antigens

Two different antigens were used in the analyses: a) the somatic extract from adult *D. immitis* worms (DiSA) prepared as Simón *et al.* (1991) (5), described and b) the recombinant form of the *Wolbachia* surface protein (rWSP) prepared according to Bazzocchi *et al.* (20). Protein concentrations were quantified with the BCA test (Pierce, USA) and the antigens stored at -80°C until use.

Measurement of specific antibodies by ELISA

IgG antibodies. To identify individuals with *D. immitis* infections all serum samples were analysed by a chromogenic ELISA with DiSA antigen following the methodology described by Simón *et al.* (5), with some modifications. In brief, the microplate wells were coated with $0.8\text{ }\mu\text{g}$ of DiSA, the serum samples were analysed at 1/100 dilution and the rabbit antihuman IgG conjugated to peroxidase (Jackson ImmunoResearch Laboratories, USA) was diluted 1/20 000.

The optical densities (OD) were measured at 492 nm in microplate reader (Bio-Rad, USA). The cutoff point (OD = 0.063) was calculated as OD arithmetical mean plus three standard deviations of group 2 sera (individuals with normal levels of total IgE).

IgE antibodies. *D. immitis* and *Wolbachia* specific IgE antibodies were analysed in representative samples from the two groups. The analysis was made using a fluoroimmunoassay (FEIA) employing UniCAP 100 and specific IgE FEIA reagents (Pharmacia Diagnostics, Sweden). Both DiSA and rWSP antigens (1 mg/mL protein) were labelled with biotin (Roche Diagnostics) at 1 : 5 molar ratio during 2 h at room temperature with vigorous shaking (500 r.p.m.). The biotinylated antigens were bound to the solid-phases by coupling 50 μL of each antigen to the Streptavidin ImmunoCAPs for 30 min and the specific IgE was measured using the UniCAP 100 system according to manufacturer's instructions (21). Results were expressed as fluorescence units (FU) of IgE antibodies, which were compared with a standard curve that allowed their conversion in IU/mL (22). The standard limit for this assay is 0.35 IU/mL that was equivalent to a response value of 193 FU.

SDS-PAGE and Western blot

DiSA proteins were separated by sodium dodecyl sulphate polyacrylamide gels of 12% (23). A total amount of 30 μg of antigenic extract was run in each lane. Proteins were transferred to polyvinyl difluoride (PVDF) membranes (Immobilon-P from Millipore, USA) using Mini Trans-Blot Cell (Bio-Rad). The membranes containing separated proteins were incubated overnight at 4°C with four different pools of sera diluted to 1/50, each of them being representative of one of the four reactivity groups, according to the *box-plots* statistical analysis (see statistical analysis). A goat antihuman IgE conjugated to alkaline phosphatase (Sigma, USA) was employed at 1/5000 dilution at room temperature for 2 h. After incubation with AP-conjugated antihuman IgE the blot was revealed with CDP-Star Chemiluminescence substrate (Roche Diagnostics). Molecular mass of the antigenic bands were calculated using the Quantity One 1-D analysis software (Bio-Rad) and molecular weight markers (SDS-7 from Sigma).

Mass spectrometry (MS) and protein identification

The proteins recognized by Western blot were analysed by MS for their identification. The protein bands from Coomassie-stained gels were excised manually and digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Germany), following the protocol of Curto *et al.* (24). Briefly, after reduction and alkylation, protein bands

were digested with modified porcine trypsin (sequencing grade from Promega, USA) at a final concentration of 8 ng/ μ L for 8 h at 37°C. Peptides were mixed with an aliquot of α -cyano-4-hydroxycinnamic acid (Bruker-Daltonics) in 33% aqueous acetonitrile and 0.25% trifluoroacetic acid; and processed for MS. This mixture was deposited onto a 600- μ m AnchorChip prestructured MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS (/MS) data were obtained in an automated analysis loop using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (25). Automated analysis of mass data was performed using the flex Analysis software (Bruker-Daltonics). The measured trypsin peptide (MALDI-MS data) and fragmented ions (MALDI-MS/MS data) masses were combined and transferred through the BioTools program (Bruker-Daltonics) to Mascot peptide mass fingerprinting (PMF) and peptide fragmentation fingerprinting (PFF) software (Matrix Science, USA) to search automatically the nonredundant protein database (NCBI nr or SwissProt) (26). MALDI-MS (/MS) spectra and database search results were manually inspected in detail using the above programs as well as home-made software. The peptide sequences matched with MS/MS data were compared with the public protein database (NCBI nr) by using the BLASTP algorithm.

Statistical analysis

The reactivity values obtained in the ELISA assays were analysed by *Mann-Whitney* test, to detect differences between groups. They were considered significant with $P < 0.05$. The reactivity value distribution for each group was showed using *box-plots*. These graphical data analysis allowed to cluster the reactivity values in *high* [higher than 3.0 times the interquartile range (IQ)], *medium* (between 1.5 and 3.0 times the IQ) and *low* (below 1.5 times the IQ). These reactivity groups correspond to the four serum-pools used in Western blot assays, as follows: pool 1 or high reactivity, pool 2 or medium reactivity and pool 3 or low reactivity; finally, pool 4 included only negative serum samples and was used as control. All statistical analyses were performed using SPSS v.14.0 software package (SPSS, USA).

RESULTS

Specific anti-*D. immitis* IgG response

To identify *D. immitis* infected individuals, all serum samples were analysed with a chromogenic ELISA for the detection of specific IgG against DiSA. The results are shown in the Figure 1. Two hundred and twenty-eight serum samples from G1 (48.8%) showed positive response, while only one serum

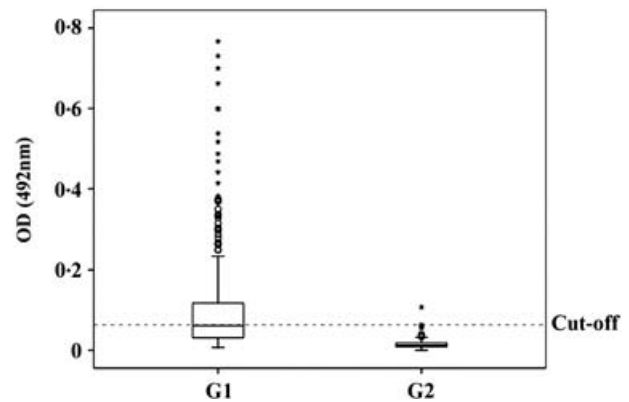


Figure 1 Box-plots graphical analysis of OD values obtained by chromoimmunoassay with DiSA (anti-DiSA IgG), with G1 (N = 467) and G2 (N = 83) sera. The reactivity groups are labelled as follows: *High reactivity and ○: medium reactivity. Cutoff = 0.063.

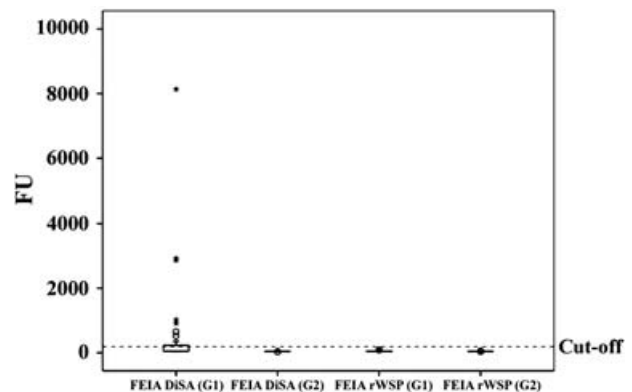


Figure 2 Box-plots graphical analysis of FU values obtained by FEIA with DiSA and rWSP respectively, using G1 (N = 42) and G2 (N = 30). The reactivity groups are labelled as follows: *High reactivity and ○: medium reactivity. Cutoff = 193.

sample from G2 exceeded the cutoff, but with a relatively low OD (0.107). Thirty-six serum samples from G1 showed medium-high reactivity ($OD > 0.247$). Statistical analysis established that the observed differences between the mean OD of the two groups were significant ($Z = 12.26$; $P < 0.0001$).

Specific IgE response against *D. immitis* and *Wolbachia* antigens

The second step was the evaluation of the specific IgE antibody response against DiSA and rWSP. FEIAs were performed in a representative selection of serum samples from G1 (42 sera) and G2 (30 serum samples). The results are shown in Figure 2. IgE positive reaction against *D. immitis*

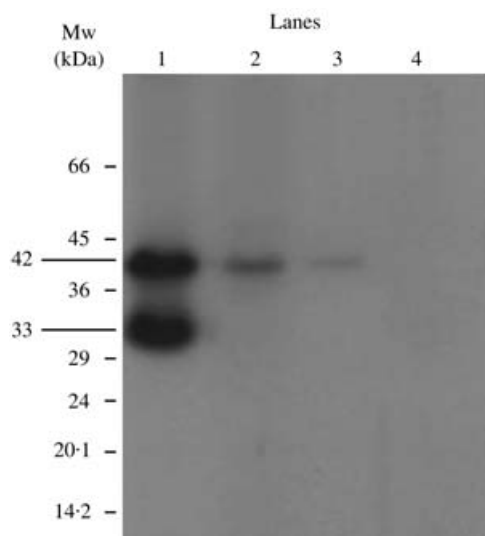


Figure 3 Western blot of Pool 1-4 specific IgE against DiSA antigens. Lane 1: Pool 1; lane 2: Pool 2; lane 3: Pool 3; lane 4: Pool 4. The major antigens of 42 and 33 kDa are indicated. Mw (kDa): SDS-7 molecular weight markers.

antigens was observed only in samples from G1 group, (28.6%, 12 out of 42), with FU between 8134 and 232. No significant levels of specific anti-*D. immitis* IgE were observed in negative individuals for specific anti-*D. immitis* IgG (G2). No specific IgE antibodies against *Wolbachia* were observed in any of the serum samples tested.

This analysis allowed us to group the serum samples in four different categories, according to their specific IgE antibody response. These groups were labelled as *high* (pool 1, FU between 8134 and 915), *medium* (pool 2, FU between 651 and 520), *low* (pool 3, FU between 357 and 232) and *negative* (Pool 4, FU between 40 and 38). All the sera grouped into pool 4, were also part of G2.

Identification of antigens of DiSA responsible for the stimulus of IgE antibodies

The next objective was to identify the molecules of DiSA that stimulate the production of specific IgE. Western blot analysis of DiSA, using the four serum pools indicated previously is shown in Figure 3. Pools exhibited different recognition pattern on polypeptides of the DiSA. The three pools with positive IgE response (pools 1–3), showed strong IgE binding against a protein band of approximately 42 kDa. Likewise, a 33 kDa fraction was strongly detected by pool 1 that grouped the highest positive values. The negative pool (pool 4) did not recognize any band on the Western blot.

Characterization of the major DiSA antigens recognized by IgE antibodies

The 42 and 33 kDa bands (Di42 and Di33, respectively) were excised from SDS gels after Coomassie staining and analysed by mass spectrometry (Figure 4). Protein identification was performed by PMF combined with PFF of selected peptides by MALDI-TOF/TOF. These analyses resulted in

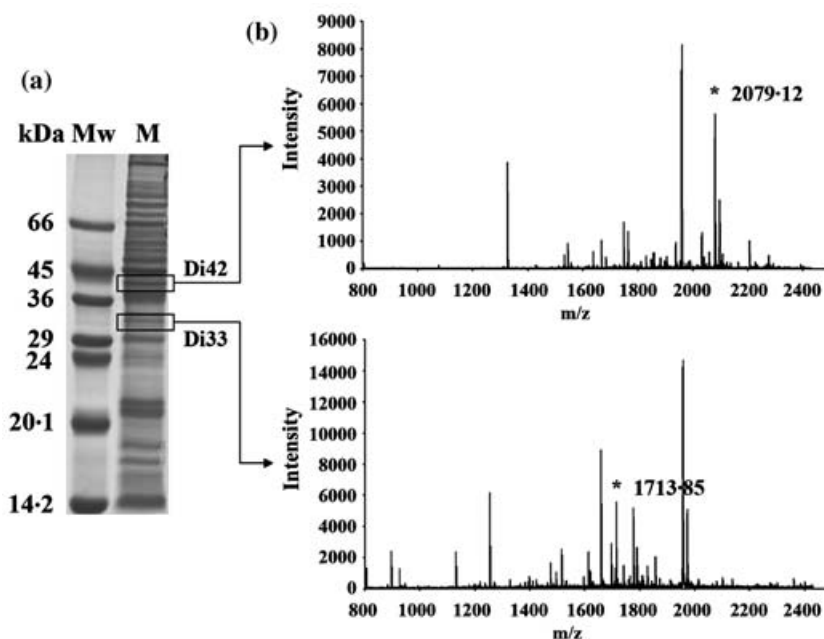


Figure 4 (a) Coomassie-stained 12% SDS-PAGE of DiSA. Di33 and Di 42 were excised and analysed by mass spectrometry. (b) MALDI-TOF spectrum of the in-gel tryptic digest of Di33 and Di42 bands isolated. Asterisk (*) indicates peaks (2079.12 and 1713.85 *m/z*) that correspond to peptides employed in the MS/MS analysis.

Table 1 Identified proteins by MS: (a) assigned band name as indicated in Figure 4, (b) identified protein and database accession numbers (NCBI nr), (c) experimental and theoretical molecular weight (kDa), (d) theoretical pI, (e) sequence of matched peptides with PMF data (*indicates peptide sequences matched with PFF data) and (f) amino acid sequence coverage for the identified proteins

Band (a)	Protein ID (Accession N ^o) (b)	Experimental-Theoretical Mw (kDa) (c)	Theoretical pI (d)	Matched peptides (e)	Sequence coverage (%) (f)
Di33	32 kDa β -galactoside-binding lectin (Galectin 1) <i>H. contortus</i> (gi 6225602)	33–32.51	6.22	SADFSGNDVPLHISVR (*) RNGDIALHFNPR NGDIALHFNPR (*) FDEKAVIRNALAANEWGNEER NEAYAFQIFVNGER LKPIGLENVEENRR	22
Di42	Fructosa-bisphosphate aldolase <i>O. volvulus</i> (gi 1945479)	42–39.48	7.75	IGATIPSHMALVEIAEVLAR (*) YASICQQHGLVPIVEPETLPDGEHDVHR KPWALTFSYGR (*)	20

two matches, showing high homologies to proteins of helminthic origin. Di33 showed homology to a 32 kDa β -galactoside-binding lectin (Galectin 1) of *Haemonchus contortus* (gi|6225602). Five peptides, with a total length of 74 amino acids, matched with Galectin 1, covering 22% of its amino acid sequence (Table 1). One of the peptide sequences obtained (FDEKAVIRNALAANEWGNEER) was aligned with the galactoside-binding lectin characteristic motif (pfam00337), belonging to the domain GLECT (cd00070). Di42 protein revealed homology to a fructose-1,6-bisphosphate aldolase (aldolase 1) of *Onchocerca volvulus* (gi|1945479), with four matching peptides comprising 73 amino acids.

Peptide sequences were compared with the public protein databases. Sequence comparison of the peptide SADFSGNDVPLHISVR from Di33 revealed a high level of identity with a *D. immitis* galectin sequence (100% galectin *D. immitis* [gi|7159326]) and with other nematode galectin sequences (100% *O. volvulus* [gi|433317], 100% *Brugia malayi* [gi|7159328], 93% *Teladorsagia circumcincta* [gi|5070261], 93% *Caenorhabditis briggsae* [gi|7159328] and 93% *Trichostrongylus colubriformis* [gi|3341813]). Similarly, the peptide IGATIPSHMALVEIAEVLAR belonging to Di42, yielded 100% of identity with a *B. malayi* aldolase (gi|158598816), and lower identity levels against no filarioidea nematode aldolases (70% *Heterodera schachtii* gi|45643643 and 70% *Globodera pallida* gi|45758650) and other groups (68% *Xenopus laevis* gi|32484316).

DISCUSSION

Human *D. immitis* infections are frequent in areas where canine dirofilariasis is endemic (7). Contacts between parasite and humans can be revealed by the detection of different isotypes of specific anti-*D. immitis* antibodies (5). On the other hand, the dual IgE and IgG recognition is frequent in patients with lymphatic filariasis (17). In this study we

demonstrate the existence of *D. immitis* infections in a group of atopic individuals (with high levels of total IgE), resident in an area of canine endemia, by the detection of specific anti-*D. immitis* IgG antibodies. Moreover, significantly higher levels of specific IgE anti-*D. immitis* antibodies were detected in a part of these individuals, suggesting a contribution of the anti-*D. immitis* IgE to the high levels of total IgE present in these group. However, specific IgE response against the rWSP of *Wolbachia* was never detected.

The analysis performed by Western blot revealed that the specific IgE response is stimulated mainly by two molecules of the antigenic extract of *D. immitis* adult worms of approximately 33 and 42 kDa. The comparison of the data obtained by mass spectrometry to these molecules and the data existing in databases, shows significant homologies of the molecule of 33 kDa with galectins-1 of *H. contortus*, *O. volvulus*, *B. malayi* and *D. immitis* (gi|6225602, gi|433317, gi|7159328 and gi|7159326), and of the molecule of 42 kDa with an aldolase of *O. volvulus* (gi|1945479). Galectins (β -galactosidase binding proteins) have been identified, not only in the species indicated, but also in other parasitic helminths like *T. circumcincta* (27) and *T. colubriformis* (28). Although *D. immitis* aldolases have not been found in the databases consulted for the mass spectrometry data, similar sequences in the genome of *D. immitis* have been identified by means of hybridization, using a specific DNA probe for an *O. volvulus* aldolase, as well as in *W. bancrofti* and *B. malayi* (29). This constitutes an evidence of the presence of this type of molecule in *D. immitis* and suggests that the aldolases seem to be well conserved in the filarial species.

Galectins and aldolases have been implicated in the host/parasite relationships. Galectin-1 is a powerful modulator of the immune response, able of suppress the expression of IL-2 e IFN- γ , maintaining the levels of IL-10 (30,31). This means the up-regulation of the Th2 response and the

down-regulation of the Th1. Moreover other types of galectins, as the galectin-3, are expressed in the surface of the eosinophils, regulating their function binding IgE to the membrane of these cells (32). Aldolases also stimulate the response of IgE antibodies (12), and they have been associated to mechanisms against the oxidative stress, because of their participation in the glycolysis (33). Moreover, in *O. volvulus* the aldolases have been located in zones related to the transformation from L3 to L4, like the cuticle, channels of connection of the oesophagus to the cuticle and in the body cavity, suggesting their participation in the molt.

Previously we have demonstrated a predominant IgE anti-*D. immitis* response in exposed individuals resident in an endemic area, without pulmonary nodules (15). Cases of clinical human dirofilariasis are associated to the presence of large amount of *Wolbachia* bacteria into the nodules and the development of a Th1-type response (34). All these data suggest that, in asymptomatic infections, a dominant IgE response stimulated mainly by a galectin and an aldolase is developed. This Th2-type response inhibits the Th1-type response. Only when the worms are trapped and destroyed in the pulmonary nodules, they release large amounts of *Wolbachia* that stimulates a Th1-type response that down-regulate the Th2 response. Whether the IgE antibodies could be responsible for the death of migrating larvae or it could be related to survival mechanisms of the parasite during the first phase of the infection, is a matter that remains to be determined. Moreover, the fact that the response of specific anti-*D. immitis* IgE stimuli for these molecules has been found in individuals with high levels of total IgE, probably indicates the contribution of the galectin and aldolase of *D. immitis* to the development of allergic reactions in individuals resident in endemic areas. Finally, we must not rule out the diagnostic or prognostic potential of the IgE produced against the galectin and aldolase in human dirofilariasis, considering that this isotype of antibodies appear almost exclusively in infected individuals without pulmonary nodules.

In conclusion, this work contributes to understand and interpretation of the immune response developed by humans exposed to *D. immitis* and stresses the possible role of this species in the development of atopic stages among resident in areas where canine dirofilariasis is present.

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