Original Article

**Dirofilaria immitis** proteins recognized by antibodies from individuals living with microfilaremic dogs

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**Abstract**

Introduction: *Dirofilaria immitis* is a nematode that affects human health in several countries of the world. This study was conducted to examine whether serum samples from the owners of microfilaremic dogs present immunoreactivity to parasite proteins.

Methodology: Eight serum samples from the owners of microfilaremic dogs were examined. Total proteins were extracted from adult worms and 12% SDS-PAGE was performed. The gel was electroblotted to a nitrocellulose membrane, and a Western blot (WB) was performed. Reactive bands of 22, 33, 39, 49, and 63 kDa in WB were excised from the gel and analyzed by mass spectrometry (MS).

Results: The MS results showed the presence of 10 different proteins of *D. immitis* recognized by the human serum samples.

Conclusions: These results indicate that in endemic areas of *D. immitis*, owners of infected dogs recognize specific proteins of the parasite, suggesting a possible infection.

**Key words:** *Dirofilaria*; filariasis; zoonotic; immunoreactivity; tropical diseases.

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**Introduction**

*Dirofilaria immitis* is a worm that affects a wide variety of mammalian hosts including humans [1,2]. The most regular lesions observed in human dirofilariasis are pulmonary nodules, which can be confused with fungal or neoplastic nodules [3]. Less frequently, *D. immitis* have been reported in subcutaneous tissue [4], testicles [5], retroperitoneal tissues [6], conjunctiva, and thorax cavity [7].

On the American continent, human infections have been reported in the United States (116 cases), Argentina, and Brazil [8]. While, serological evidence of infection in humans was reported in Colombia [9,10]. In Mexico, *D. immitis* infection in dogs has mainly been reported in southeastern states such as Yucatán [11] and Tabasco [12]. Although, a recent study carried out in the central area of country (Puebla, Mexico), reported the presence of antibodies against *D. immitis* in dogs and humans of this state [13]. However, knowledge of human infections is lacking, and the damages to human health caused by this parasite may be potentially underestimated in these endemic regions.

Information related with *D. immitis* infections in people living endemic areas of Mexico could be used to include this zoonoses in differential diagnoses when lung lesions are found in patients from endemic locations. Therefore, the goal of present study was to identify immunoreactivity against the proteins of *D. immitis* in the sera of individuals who owned microfilaremic dogs in a rural locality of Tabasco, Mexico.
Methodology

Study area

The present study was performed in March of 2018 in Cupilco in the municipality of Comalcalco, Tabasco, Mexico. Cupilco (population of ~945 inhabitants) is located at 18° 13' N and 93° 08' W at an average elevation of approximately 5 m [14].

Ethical statement

This study was approved by the Ethical Committee of University in State of Tabasco, Mexico (Approval N° 0191). Humans and dogs sampled in this study, biological samples and residues were treated and handled in accordance with federal regulations in Mexico (NOM-046-zoo-1995, NOM-087-ECOL-SSA1-2002) and following the recommendations emitted by Ethical Committee.

Canine blood sample collection

Blood samples were collected from the cephalic vein of each dog using the Vacutainer™ tubes containing EDTA as an anticoagulant. Serum samples were collected using tubes without anticoagulant.

DNA extraction from canine blood samples

DNA extraction was performed according to the salting-out technique described by Miller et al. [15] with several modifications as describes Torres-Chable et al. [12]. The DNA pellet was dried for 30 minutes and suspended in 50 µl of TE solution (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]).

PCR description to identify zoonotic filarial species in dogs

We used pan-filarial forward primer DIDR-F1 and reverse primer DIDR-R1 designed to amplify different DNA fragments of several filarial nematodes [16]. Positive samples were evaluated again using the specific primers DI COI-F1 and DI COI-R1 to identify D. immitis and AR COI-F1 and AR COI-R1 primers to discard the possible presence of A. reconditum [16]. The PCR reactions were performed using 2.5 µL of genomic DNA (50–200 ng) following the methodology described by Torres-Chable et al. [12]. PCR products were examined by 2% agarose gel electrophoresis.

Human population and blood sample collection

Following the positive diagnosis of dogs, a second sampling was carried out to collect serum samples from the dog owners. Blood samples from seven women and one man aged 44 to 78 years old were collected in Vacutainer™ tubes without anticoagulant. Samples were centrifuged at 2000 ×g for 15 minutes, sera were recovered, placed in new serological tubes (1.5 mL) and frozen at −20 °C until use.

Total soluble protein extracts from D. immitis

Adults of D. immitis collected directly from the heart of a dog during necropsy in the Veterinary Hospital of the Universidad Juarez Autonoma de Tabasco in 2016 (five females and four males) were used for protein extraction. Parasites preserved in freezing at -80 °C were thawed and washed in phosphate-buffered saline (0.01 M PBS [pH 7.4]) and subsequently placed in a sterile mortar. Approximately 50 mL of liquid nitrogen was added to the mortar to facilitate the maceration of the parasites. Subsequently, 500 µL of protein extraction solution (50 mM Tris-HCl [pH 7.4], 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 µg/mL of leupeptin, and 1 µg/mL of aprotinin) were added, and the maceration was continued. The macerated material was centrifuged at 15,000 ×g for 15 minutes at 4 °C. The supernatant was recovered and placed in a sterile serological tube. Recovered proteins were quantified using the Bradford technique [17].

SDS-polyacrylamide gel elaboration

A 12% SDS-PAGE was initially performed using 30 µg of the total extract of proteins to observe the integrity of the extracted protein from D. immitis. Subsequently, the 12% SDS-PAGE was performed using 5 µg of the protein extract at 80 V in a Miniprotein™ II electrophoresis cell (Bio-Rad Laboratories, Inc., Philadelphia, USA). The gel was electroblotted to a nitrocellulose membrane overnight.

Western blot for identification of D. immitis proteins in human serum

The nitrocellulose membrane was cut into strips. Each strip was placed in a sterile tube (15 mL), and blocked overnight with 5 mL of 5% non-fat dried milk in 1X PBS under constant agitation (30 rpm). Subsequently, the strips were washed with a solution of 1X PBS containing 1% Tween 20 under constant agitation at 30 rpm (washing step).

The primary incubation was performed in sterile tubes (15 mL) with a solution of human serum and 1X PBS at distinct dilutions (1:10, 1:50, 1:100, 300, 1:500, 1:700, and 1:1000) to evaluate the best dilution to use in the WB. The primary incubation was conducted overnight under constant agitation (30 rpm), and the washing step was then repeated. A canine serum
sample positive for *D. immitis* was also incubated and used as a positive canine control (CC+).

The secondary incubation was conducted using the goat anti-human IgG antibody linked with horseradish peroxidase (HRP) (Kirkegaard and Perry Laboratories, KPL, Gaithersburg, Maryland, USA) in a 1:400 dilution according to the supplier’s instructions. The secondary incubation of CC+ was performed using the Goat Anti-Canine IgG linked to HRP (Kirkegaard and Perry Laboratories, KPL, Gaithersburg, Maryland, USA) under similar conditions of human serum samples. A strip without primary incubation (only with secondary incubation) was used as a non-specific binding control (NSBC). A serum sample which was negative for several mosquito-borne and vector-borne diseases was used as a negative human control (HC−).

Finally, the detection of immunoreactive bands was carried out using diaminobenzidine (DAB) as a substrate of HRP. A tablet containing 10 mg of DAB was dissolved in 10 mL of 1X PBS, and 100 µL of 3% hydrogen peroxide was added at moment of the reaction. The strips were submerged in the solution for 5 seconds and were subsequently submerged in sterile water to stop the reaction.

**Protein digestion and MS sample preparation**

The performed WB was used to identify the reactive bands containing immunogenic proteins which were excised from a new 12% SDS-PAGE and sent to the Proteomics Unit of the Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional (Irapuato, Mexico) for identification by MS. The protein bands were digested in gel according to the trypsin digestion method [18]. Subsequently, the supernatants containing the peptide mixtures were collected and dried in a SpeedVac™ vacuum concentrator (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The peptide mixtures were then analyzed by MS.

**Nanoflow LC-MS/MS**

All experiments were performed using ultra-performance liquid chromatography (nanoACQUITY system™, Waters™, Location) coupled with a linear ion trap-mass spectrometer (LTQ velos™, Thermo Fisher Scientific, Bremen, Germany) equipped with a nano-electrospray ion source. Solvent A consisted of 0.1% FA and solvent B of 100% ACN in 0.1% FA. Three µl of trypically digested proteins were bound to a pre-column (Symmetry™ C18, 5 µm, 180 µm × 20 mm, Waters) according to Shevchenko *et al.* [18]. Subsequently, the flow was switched to a 10-cm capillary UPLC column (100 µm ID BEH-C18, 1.7 µm particle size). The column temperature was controlled at 35 °C. The peptides were separated by a 60-minutes gradient method at a flow rate of 400 nl/minute. The gradient was programmed as follows: 3–50% solvent B (over 30 minutes), 50–85% B (over 2 minutes), 85% B (over 4 minutes), and 3% B (over 22 minutes). The peptides were eluted into the nano-electrospray ion source using a standard coated silica tip (NewObjective, Woburn, Massachusetts, USA). The mass spectrometer was operated in data-dependent acquisition mode in order to automatically alternate between full scan (400–1600 m/z) and the subsequent Top 5 MS/MS scans in the linear ion trap with dynamic exclusion enabled. Collision-induced dissociation was performed using helium as a collision gas at normalized collision energy of 35% and activation time of 10 milliseconds. Data acquisition was controlled by the Xcalibur 2.0.7 software (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**Automated data evaluation**

Tandem mass spectra were extracted in Proteome Discoverer version 1.4 and searched in Sequest against a NCBI *Dirofilaria* database (413 entries). Searches were executed with the following parameters: 2 Da parent MS ion window, 1 Da MS/MS ion window, and two missed cleavages allowed. The iodoacetamide derivative of cysteine (carbamidomethyl-cysteine) was specified in Sequest as a fixed modification and the oxidation of methionine as a variable modification.

**Figure 1.** SDS-PAGE at 12% where can see the protein profile of the total protein extract of adults *Dirofilaria immitis*. Lane 1: Molecular weight marker (Precision Plus Protein™, Dual Colors Standards, Bio Rad, Ciudad de Mexico, Mexico). Lanes 2-10: 30 µg of total protein extract of adults *Dirofilaria immitis* by lane.
Results

Thirty-four dogs were sampled in the locality of Cupilco, Tabasco, Mexico. *Dirofilaria immitis* was the only species found, with a prevalence of 26.47% (9/34 dogs). Eight human sera from the owners of positive dogs were examined and used to perform the WB analysis.

The polyacrylamide gel showed abundant, well-defined bands between 16 and 238 kDa (Figure 1). Subsequently, the WB showed three bands of ~22, ~33, and ~39 kDa. Two individuals (34H and 35H) recognized two bands of 49 and 63 kDa. These bands remained stable despite the different dilutions, although the human sera used in 1:500 dilution generated the best visual results in WB (Figure 2).

The ~33 and ~39 kDa bands were recognized by all of the evaluated human sera. These bands were not recognized in the NSBC and HC− controls. In contrast, the band of ~22 kDa presented non-specific recognition because it was also recognized in the NSBC. Reactive bands were cut and subsequently analyzed by MS. Table 1 shows the identity of sequenced proteins. The results of the MS analysis showed that the individuals who owned dogs positive for *D. immitis* infection with active microfilaremia are able to recognize somatic proteins of this parasite.

Discussion

Dirofilariasis caused by *D. immitis* is a zoonoses that is increasingly reported worldwide [6-8]. In Mexico, there is only one previous study that reports serological evidence of human infections, despite the presence of infected dogs and vectors. Infections in humans are characterized by pulmonary lesions and a greater immune response than in dogs [13,19,20].

Table 1. Antigenic protein bands of *D. immitis* recognized by sera from individuals who own dogs with heartworm disease (identified by mass spectrometry).

| Band in Gel | Accession code | Sequence description | Species | MW (kDa) | IP | Sequence coverage | Mascot score |
|------------|----------------|----------------------|---------|---------|----|------------------|-------------|
| 63 kDa     | AAC24752.1     | Transglutaminase precursor | *Dirofilaria immitis* | 57.2 | 5.9 | 6.24 | 8.28 |
| 63 kDa     | AEA91534.1     | 2,3-diphosphoglycerate-independent phosphoglycerate mutase | *Dirofilaria immitis* | 57.1 | 6.49 | 16.31 | 34.56 |
| 49 kDa     | AAC13548.1     | Beta-tubulin         | *Onchocerca volvulus* | 50.1 | 4.9 | 21.88 | 54.27 |
| 39 kDa     | AAD03405.2     | Calreticulin precursor | *Dirofilaria immitis* | 43.3 | 5.24 | 11.53 | 9.75 |
| 39 kDa     | AFL46380.1     | Actin                | *Dirofilaria immitis* | 41.8 | 5.48 | 22.61 | 37.92 |
| 39 kDa     | BAA02004.1     | Neutrophil chemotactic factor DiNCF precursor | *Dirofilaria immitis* | 38.9 | 7.65 | 20.24 | 11.9 |
| 39 kDa     | AFL46382.1     | Glyceraldehyde 3 phosphate dehydrogenase | *Dirofilaria immitis* | 36.2 | 7.5 | 91.15 | 1631 |
| 33 kDa     | AAF37720.1     | Galectin             | *Dirofilaria immitis* | 32.0 | 6.4 | 53.38 | 396.80 |
| 22 kDa     | AAC47103.1     | Pepsin inhibitor Dit 33 | *Dirofilaria immitis* | 26.0 | 8.0 | 60.26 | 91.96 |
| 22 kDa     | AAD11968.1     | P22U                 | *Dirofilaria immitis* | 23.5 | 8.4 | 16.04 | 10.18 |

MW: Molecular weight; IP: Isoelectric point.
Beta-tubulin is a structural protein of eukaryotic cells [24]. This protein has not been previously reported in infections in dogs or humans. In this study, it was weakly recognized by two of the individuals evaluated (band ~49 kDa). Therefore, it does not appear to be important in infections caused by *D. immitis* in humans.

Four proteins were identified from the observed band ~39 kDa in WB: Calreticulin precursor (43.3 kDa by MS), actin (41.8 kDa by MS), neutrophil chemotactic factor DiNCF precursor (38.9 kDa, by MS) and glyceraldehyde 3 phosphate dehydrogenase (36.2 kDa by MS). Calreticulin precursor participates in the Ca\(^{2+}\) accumulation and protein synthesis [25]. The actin of *D. immitis* is involved in the motility process, binds to plasminogen [26], and interacts with other proteins to induce the activation of the fibrinolytic system in dogs [27]. This protein has been recognized in human infections [20,23,27]. Neutrophil chemotactic factor DiNCF precursor was another protein recognized by human sera in the present study. However, no previous reports have been published on this protein in human infections. Glyceraldehyde-3-phosphate dehydrogenase is involved in the glycolysis and as a plasminogen receptor in *D. immitis*, thus contributing to prevent the formation of clots in the intravascular environment, facilitating the survival of nematode [28]. Since calreticulin precursor and the neutrophil chemotactic factor DiNCF precursor have not been reported in human infections, the immune response observed in the band ~39 kDa in WB in this study could really be originated by actin and glyceraldehyde 3 phosphate dehydrogenase. Therefore, in future studies, these proteins should be evaluated individually.

The band ~33 kDa, was the clearest and the most constant band observed in the WB performed. Galectin was the only protein identified from this band. In dogs infected with *D. immitis*, galectin contribute to avoid intravascular clots [28]. Previously, this protein has been identified as responsible for the specific IgE response in humans exposed to *D. immitis* [29]. Galectin is scattered throughout the soma, but is especially abundant in the cuticles of adult parasites [29] and microfilariae [23]. So, it is possible that the circulating antibodies in humans living in endemic areas of canine filariasis are due to the constant inoculation of microfilariae during mosquito bites, although this possibility should be evaluated.

Pepsin inhibitors Dit 33 (26 kDa, by MS) and P22U (23.5 kDa, by MS) were identified from a band of ~22 kDa observed in WB. Both proteins were recognized by patients with pulmonary dirofilariasis in a previous study [20]. Pepsin inhibitor Dit 33 inhibits protease activity and is considered a specific marker of feline heartworm disease [30]. P22U is excreted when L3 larvae of *D. immitis* molt into L4 larvae and it relates to the synthesis of isoprenoids [31]. However, the band of ~22 kDa showed a non-specific immune response in WB. Therefore, pepsin inhibitors Dit 33 and P22U proteins must be individually evaluated to identify the protein responsible for the non-specific response observed in WB.

Despite the presence of endosymbiont *Wolbachia* bacteria associated with adult filariae *Wolbachia* proteins were not detected in this study. Similar results were found by González-Miguel et al. [20], presumably due to the detection limit associated with the WB technique.

Unfortunately, the medical staff in charge did not admit the taking of thoracic x-ray plates, which limits the results found. However, the authors consider this study to be the first reference of *D. immitis* infection in inhabitants of Tabasco, Mexico. In addition, this study provides information from specific proteins recognized by individuals living with microfilaremic dogs. Future studies should integrate a greater number of exposed individuals, x-rays to identify pulmonary nodules and the individual evaluation of reactive proteins in order to know the best biomarkers to identify infections caused by *D. immitis*, avoiding unnecessary surgeries.

**Conclusions**

Individuals who owned microfilaremic dogs presented immunoreactivity against of specific proteins of *D. immitis*. Pulmonary lesions should be clinically diagnosed in these individuals using x-rays. Immunoreactive proteins found in this study should be studied more extensively in order to know the best biomarkers to identify infections caused by *D. immitis*, avoiding unnecessary surgeries when pulmonary lesions will be found on x-rays.

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