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Original Article

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

Oswaldo M Torres-Chable[1], Ligia G Brito-Argaez[2], Ignacio R Islas-Flores[2], Claudia V Zaragoza-Vera[1], Maritza Zaragoza-Vera[1], Guadalupe Arjona-Jimenez[1], Carlos M Baak-Baak[3], Nohemi Cigarroa-Toledo[4], Roberto Gonzalez-Garduño[5], Carlos I Machain-Williams[3], Julian E Garcia-Rejon[3]

[1] División Académica de Ciencias Agropecuarias, Laboratorio de Enfermedades Tropicales y Transmitidas por Vectores, Universidad Juárez Autónoma de Tabasco, Villahermosa, Tabasco, México
[2] Laboratorio de Bioquímica y Biología Molecular, Centro de Investigaciones Científicas de Yucatán (CICY), Mérida, Yucatán, México
[3] Laboratorio de Arbovirología, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Yucatán, México
[4] Laboratorio de Biología Celular, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Mérida, Yucatán, México
[5] Unidad Regional Universitaria Sursureste, Universidad Autónoma Chapingo, Teapa, Tabasco, México

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 Yucatan [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-R1designed 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 ºC 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 Miniprotean™ 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, 1:300, 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.

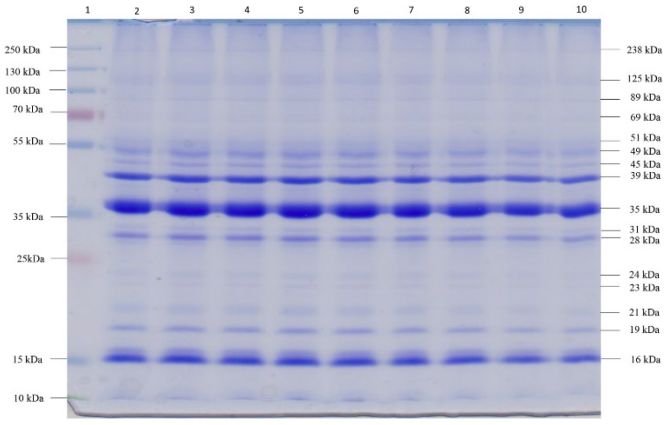
Nanoflow LC-MS/MS

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 tryptically 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 ProteinTM, Dual Colors Standards, Bio Rad, Ciudad de Mexico, Mexico). Lanes 2-10: 30 µg of total protein extract of adults *Dirofilaria immitis* by lane.

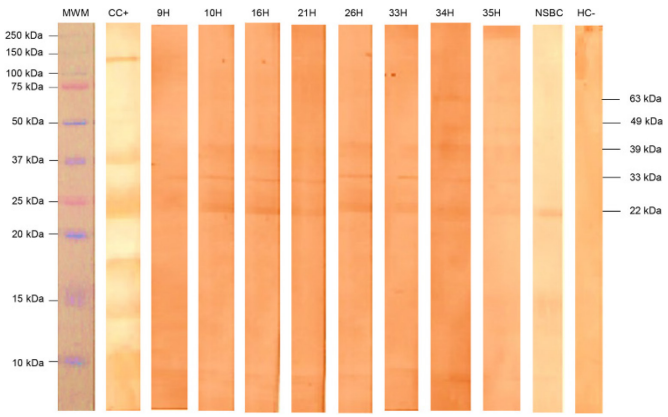
Results

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.

Figure 2. Western blot of human sera evaluated that shows reactive bands to *D. immitis*.



MWM: Molecular weight marker (Precision Plus ProteinTM, Dual Colors Standards, Bio Rad, Mexico). CC+: Positive canine control. 9H-35H Human sera evaluated. NSBC: Non-specific binding control. HC-: Negative human control.

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].

In this study, 10 specific proteins of *D. immitis* were recognized by human sera evaluated. Two individuals recognized a band of ~63 kDa. Results of MS of this band showed to the transglutaminase precursor and 2,3-diphosphoglycerate-independent phosphoglycerate mutase, both enzymes with a molecular weight of 57 kDa. Transglutaminase precursor has redox activities and cross-linking proteins that filariae use to synthesize its sheath, cuticle, and epicuticle [21,22]. The enzyme 2,3-diphosphoglycerate-independent phosphoglycerate mutase is involved in the glycolysis of the parasite. Both enzymes were recognized by dogs and humans with pulmonary dirofilariasis in previous studies [20,23]. Therefore, the two individuals who recognized these enzymes could have developed pulmonary nodules despite being asymptomatic.

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	6.4	53.38	396.80
22 kDa	AAC47103.1	Pepsin inhibitor Dit 33	*Dirofilaria immitis*	26	8	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 L_3 larvae of *D. immitis* molt into L_4 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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References

1. Furtado AP, Do Carmo ES, Giese EG, Vallinoto AC, Lanfredi RM, Santos JN (2009) Detection of dog filariasis in Marajo Island, Brazil by classical and molecular methods. Parasitol Res 105: 1509-1515.
2. Genchi C, Kramer L, Prieto G (2001) Epidemiology of canine and feline dirofilariasis: a global view. Simón F, Genchi C

Editors. Heartworm infection in humans and animals. Edited by Salamanca, Spain: University of Salamanca editions. 121-134.
3. Kang HJ, Park YS, Lee CH, Lee SM, Yim JJ, Yoo CG, Kim YW, Han SK, Chai JY, Lee J (2013) A case of human pulmonary dirofilariasis in a 48-year-old Korean man. Korean J Parasitol 51: 569-572.
4. Foissac M, Million M, Mary C, Dales JP, Souraud JB, Piarroux R, Parola P (2013) Subcutaneous infection with *Dirofilaria immitis* nematode in human, France. Emerg Infect Dis 19: 171-172.
5. Theis JH, Gilson A, Simon GE, Bradshaw B, Clark D (2001) Case report. Unusual location of *Dirofilaria immitis* in a 28-year-old man necessitates orchiectomy. Am J Trop Med Hyg 64: 317-322.
6. Pampiglione S, Rivasi F, Gustinelli A (2009) Dirofilarial human cases in the Old World, attributed to *Dirofilaria immitis*: a critical analysis. Histopathology 54: 192-204.
7. Pozgain Z, Dulic G, Sego K, Blazekovic R. (2014) Live *Dirofilaria immitis* found during coronary artery bypass grafting procedure. Eur J Cardiothorac Surg 46: 134-136.
8. Simon F, Siles-Lucas M, Morchon R, Gonzalez-Miguel J, Mellado I, Carreton E, Montoya-Alonso JA (2012) Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin Microbiol Rev 25: 507-544.
9. Vieira C, Velez ID, Montoya MN, Agudelo S, Alvarez MI, Genchi C, Simon F (1998) *Dirofilaria immitis* in Tikuna Indians and their dogs in the Colombian Amazon. Ann Trop Med Parasitol 92: 123-125.
10. Vieira C, Montoya M, Agudelo S, Velez ID, Simon F (2000) Human antibody response to a 56-kDa purified excretory/secretory product of *Dirofilaria immitis*. Trop Med Int Health 5: 855-859.
11. Rodríguez V, Domínguez A, Solís R, Cob G (1994) Prevalence of *Dirofilaria immitis* in stray dogs in the city of Merida, Yucatan, Mexico. Vet Méx 25: 145-148.
12. Torres-Chable OM, Baak-Baak CM, Cigarroa-Toledo N, Blitvich BJ, Brito-Argaez LG, Alvarado-Kantun YN, Zaragoza-Vera CV, Arjona-Jimenez G, Moreno-Perez LG, Medina-Perez P, Machain-Williams CI, Garcia-Rejon JE (2018) Molecular detection of *Dirofilaria immitis* in dogs and mosquitoes in Tabasco, Mexico. J Vector Borne Dis 55: 151-158.
13. Zumaquero JL, Simón F, Carretón E, Hernández I, Sandoval-Ruiz C, Morchón-García, R (2020) Prevalence of canine and human dirofilariosis in Puebla, Mexico. Vet Parasitol 282: 109098
14. National Institute of Statistics, Geography and Informatics-INEGI. Mexico (2018). Municipal geographic information of the United Mexican States. Tabasco. Available: http://www.inegi.org.mx. Accessed: 13 October 2018.
15. Miller S, Dykes D, Polesky H (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16: 1215.
16. Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Dominguez Alpizar JL (2006) Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet Parasitol 135: 303-314.
17. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-54.
18. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1: 2856-2860.
19. Grieve RB, Gebhardt BM, Bradley RE, Sr. (1979) *Dirofilaria immitis*: cell-mediated and humoral immune responses in experimentally-infected dogs. Int J Parasitol 9: 275-279.
20. González-Miguel J, Rosario L, Rota-Nodari E, Morchón R, Simón F (2010). Identification of immunoreactive proteins of *Dirofilaria immitis* and *D. repens* recognized by sera from patients with pulmonary and subcutaneous dirofilariosis. Parasitol Int 59: 248-256.
21. Rao RU, Mehta K (2004) Transglutaminases, thioredoxins and protein disulphide isomerase: diverse enzymes with a common goal of cross-linking proteins in lower organisms. Indian J Exp Biol 42: 235-243.
22. Eckert RL, Kaartinen MT, Nurminskaya M, Belkin AM, Colak G, Johnson GVW, Mehta K (2014) Transglutaminase regulation of cell function. Physiol Rev 94: 383-417.
23. Sassi AJ, Geary JF, Leroux LP, Moorhead AR, Satti M, Mackenzie CD, Geary TG (2014) Identification of *Dirofilaria immitis* proteins recognized by antibodies from infected dogs. J Parasitol 100: 364-367.
24. Nogales E, Wolf SG, Downing KH (1998) Correction: Structure of the alpha beta tubulin dimer by electron crystallography. Nature 391:199-203.
25. Jiang Y, Dey S, Matsunami H (2014) Calreticulin: Roles in cell-surface protein expression. Membranes 4: 630-641.
26. Lind S, Smith CJ (1991) Actin accelerates plasmin generation by tissue plasminogen activator. J Biol Chem 266: 17673-17678.
27. González-Miguel J, Morchón R, Carretón E, Montoya-Alonso JA, Simón F (2013) Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system. Vet Parasitol 196: 235-240.
28. Gonzalez-Miguel J, Morchon R, Siles-Lucas M, Oleaga A, Simon F (2015) Surface-displayed glyceraldehyde 3-phosphate dehydrogenase and galectin from *Dirofilaria immitis* enhance the activation of the fibrinolytic system of the host. Acta Trop 145: 8-16.
29. Pou-Barreto C, Quispe-Ricalde MA, Morchon R, Vazquez C, Genchi M, Postigo I, Valladares B, Simon F (2008) Galectin and aldolase-like molecules are responsible for the specific IgE response in humans exposed to *Dirofilaria immitis*. Parasite Immunol 30: 596-602.
30. Hong XQ, Santiago Mejia J, Kumar S, Perler FB, Carlow CK (1996) Cloning and expression of DiT33 from *Dirofilaria immitis*: a specific and early marker of heartworm infection. Parasitol 112: 331-338.
31. Frank GR, Wisnewski N, Brandt KS, Carter CR, Jennings NS, Selkirk ME (1999) Molecular cloning of the 22-24 kDa excretory-secretory 22U protein of *Dirofilaria immitis* and other filarial nematode parasites. Mol Biochem Parasitol 98: 297-302.

Corresponding author

Oswaldo Margarito Torres-Chable. PhD.
Laboratorio de Enfermedades Tropicales y Transmitidas por Vectores. División Académica de Ciencias Agropecuarias, Universidad Juarez Autonoma de Tabasco. Calle Centenario s/n. Colonia Reforma, Villahermosa, Tabasco, Mexico.
Tel: (993) 3581500 Ext. 6602
Fax: (993)581585
E-mail: oswaldo.torres@ujat.mx

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