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Heartworm (*Dirofilaria immitis*) Prevalence in Dogs Determined by In-House ELISA Based on Filaria-Specific Antibodies in Tropical and Temperate Regions of Mexico

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Abstract: *Dirofilaria immitis* is a mosquito-borne nematode of dogs, other carnivores and, occasionally, humans. Globally, *D. immitis* infection (which causes heartworm) is typically more prevalent in tropical than temperate regions. In this study, the seroprevalence of *D. immitis* was determined from a sample of 335 non-stray dogs from four municipalities, two each from the states of Puebla and Guerrero in Mexico, using polyclonal antibodies to detect serum antigens using an enzyme-linked immunosorbent assay (ELISA). The accuracy of the assay was compared with the modified Knott's test. The polyclonal antibody used in the direct ELISA had a high sensitivity (100%) with variable specificity (98.2–98.8%) in the municipalities of Puebla and Guerrero. The area under the curve for the four municipalities was 1.0, indicating a high accuracy test, with a cut-off value ranging from 0.45 to 0.50. The overall prevalence of *D. immitis* infection was 17.56% (59 out of 335). The highest prevalence was in Acapulco (24.78%), followed by Chilpancingo (20.93%), Tecamachalco (10.81%) and Quecholac (8.06%). The highest percentage of positive samples was detected in tropical regions (23.12%) and the lowest in temperate regions (9.56%). This study demonstrates that polyclonal anti-*D. immitis* antibodies can successfully diagnose heartworm-infected dogs and be used to monitor prevalence effectively and develop prevention strategies against *Dirofilaria* infection.

Keywords: canine antigen test; canine heartworm; dogs; *Dirofilaria immitis*; epidemiology; ELISA; prevalence

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1. Introduction

Dirofilaria immitis is a mosquito-borne, filarial nematode with worldwide distribution and is endemic on six continents [1,2]. Canine heartworm disease caused by *D. immitis* is particularly prevalent in tropical and temperate regions, while *D. repens* (subcutaneous infection) occurs in the Old World [1,3]. Of all the *Dirofilaria* species, *D. immitis* and *D. repens* are of medical and veterinary concern due to their high incidence and prevalence.

Furthermore, they are the most pathogenic nematodes, causing mild to severe disease in a wide variety of mammals, including dogs, cats and humans [4–6].

Dirofilaria immitis typically inhabits the right ventricle and pulmonary arteries of its hosts and presents as a persistent cough, laboured breathing, physical activity intolerance and a loss of appetite and weight [7,8]. Several mosquito species of the genera *Aedes*, *Culex* and *Anopheles*. have been reported as competent vectors that use the same definitive hosts, primarily canids [9,10].

There is a lack of information regarding the prevalence of canine dirofilariasis in different regions of Mexico with varying climatic conditions. However, comparative data concerning the prevalence of dirofilariasis in other regions with various climates are available. The prevalence of dogs infected with *D. immitis* in Mexico ranges from 2.44% to 59.8% [11–15]. However, this prevalence varies dramatically between climatic regions, as does the distribution and density of the mosquito vector species and fertility, the techniques used for diagnoses, age of the host, length of the hair coat, predominant hair colour, living conditions and the ease of movement of dogs between locations [16–18].

In the last two decades, there has been a significant increase in the geographic distribution of canine heartworm infections. Studies have focused on the diagnosis and prevalence of *D. immitis* in domesticated dogs and wild canines [19], as the rapid, accurate diagnosis of infection is vital for the epidemiology, surveillance and control of the disease. The diagnosis of heartworm infection in dogs is typically determined using a combination of the modified Knott's test for the identification of microfilariae in blood samples and an enzyme-linked immunosorbent assay (ELISA) for the detection of antigens associated with the presence of adult female *D. immitis*. In this study, we evaluated the performance of a polyclonal anti-*D. immitis* antibody (produced using protein antigens from adult female filarial parasites) in ELISA-based serodiagnoses to determine the prevalence of *D. immitis* infection in dogs in two different climatic regions of Mexico.

2. Results

2.1. Determination of Positive and Negative Samples via Direct ELISA

Of the 50 samples obtained from the State of Guerrero, 14 were positive according to the modified Knott's test and confirmed using SNAP 4Dx Plus. The ROC cut-off point was established at 0.35, defined as the mean value +3 standard deviations (SDs) of the 36 negative serum samples obtained from dogs from Guerrero that were negative for microfilariae using the modified Knott's test and confirmed using a qualitative ELISA test (SNAP 4Dx Plus, IDEXX Laboratory, Westbrook, ME, USA). The mean optical density (OD) of the positive sera was 0.80 with an SD of 0.08; the highest value was 1.05, and the lowest was 0.71. The mean OD of the 36 negative sera samples was 0.38, with an SD of 0.11. The sample with the highest OD was 0.49, and the lowest was 0.18.

2.2. Prevalence of D. immitis and the ROC Analysis

A total of 335 dog were sampled from two states and four municipalities in Mexico for use in the study. The prevalence of heartworm infection based on direct ELISA was 17.56% (59 out of 335). A summary of *D. immitis* prevalence results is presented in Table 1. The highest prevalence occurred in Acapulco (a tropical climate), with 28 of 113 samples testing positive (24.78%), and the lowest was observed in Quecholac (a temperate climate), with 5 of 62 samples testing positive (8.06%). The highest prevalence among the two climate areas was in the tropical region (23.12%, 46 out of 199), followed by the temperate region (9.56%, 13 out of 136). Overall, there was a significant difference in heartworm prevalence between Puebla's temperate climate and Guerrero's tropical one ($p = 0.001$).

Table 1. Prevalence of *Dirofilaria immitis* in dogs of Mexico in two climate classifications (temperate and tropical) analysed by direct enzyme-linked immunosorbent assay (ELISA) using a polyclonal antibody to detect parasite antigens (n = 335).

State/District/Municipality	Overall		Negative Samples (OD < 0.45)		Positive Samples (OD ≥ 0.45)		
	No. of Dogs	Mean OD ± SD	No. of Samples	Mean OD ± SD	No. of Samples	Mean OD ± SD	Prevalence (%)
Puebla state							
Tecamachalco/Quecholac	62	0.24 ± 0.13	57	0.21 ± 0.05	5	0.66 ± 0.01	8.06 (3.49–17.53)
Tecamachalco/Tecamachalco	74	0.29 ± 0.18	66	0.23 ± 0.06	8	0.75 ± 0.09	10.81 (5.58–19.91
Guerrero state							
Las Vigas/Acapulco	113	0.35 ± 0.24	85	0.23 ± 0.08	28	0.71 ± 0.15	24.78 (17.74–33.48)
Chilpancingo/Chilpancingo	86	0.34 ± 0.26	68	0.21 ± 0.07	18	0.78 ± 0.12	20.93 (13.67–30.68)
Climate regions							
Temperate (Puebla)	136	0.27 ± 0.16	123	0.22 ± 0.06	13	0.72 ± 0.08	9.56 [a] (5.67–15.67)
Tropical (Guerrero)	199	0.34 ± 0.24	153	0.21 ± 0.07	46	0.74 ± 0.16	23.12 [a] (17.80–29.45)
Overall	335	0.31 ± 0.21	276	0.22 ± 0.06	59	0.73 ± 0.15	17.56 (13.91–28.29)

OD, optical density; SD, standard deviation. Column with common superscript differ significantly ($p < 0.05$).

The ROC curve analysis for *D. immitis* antigen detection exhibited high accuracy with an AUC of 1.0 for the four municipalities studied. In our evaluation of the diagnostic utility of an ELISA test using a polyclonal antibody to detect *D. immitis* parasite antigens, a sensitivity of 100% was observed across all the municipalities. The highest specificity (98.8%) was obtained for the serum samples from Acapulco, followed by the states of Tecamachalco (98.5%) and Chilpancingo (98.5%). Lower specificity values (98.2%) were observed in the serum samples from Quecholac. The highest cut-off value was for Tecamachalco (0.50), followed by Quecholac (0.49) and Acapulco (0.49) and Chilpancingo (0.45, Figure 1).

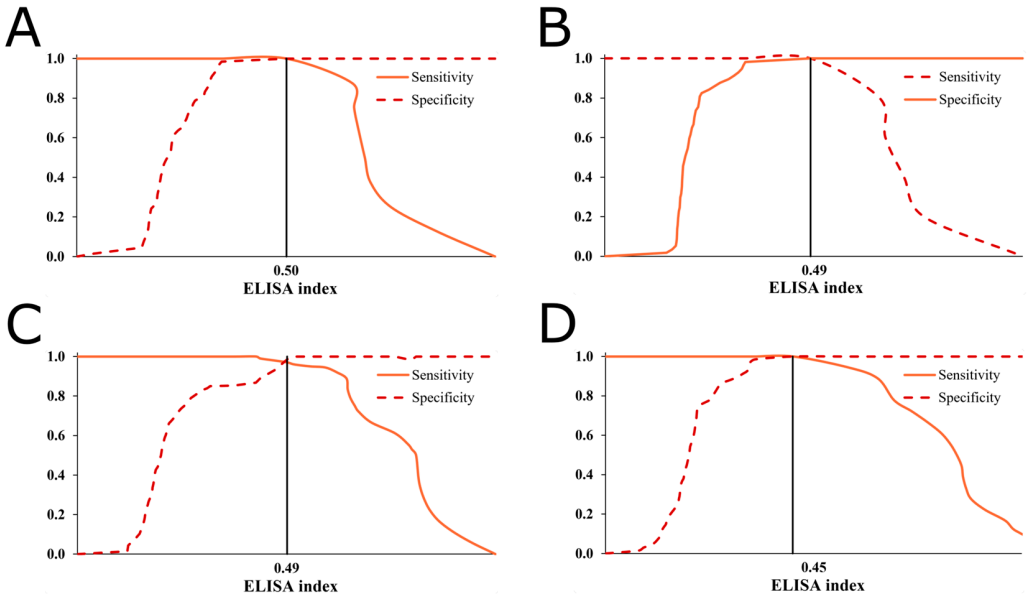

Figure 1. Optimisation of cut-offs using a direct ELISA to detect *Dirofilaria immitis* parasite antigens in Tecamachalco (**A**), Quecholac (**B**), Acapulco (**C**) and Chilpancingo (**D**).

3. Discussion

In this study, we developed a direct ELISA test to determine serum antigens of *D. immitis* in domestic dogs based on polyclonal antibodies produced with protein antigens from adult female filarial worms. The estimated prevalence of *D. immitis* across both Mexican states with different climatic regions was 17.56%.

There are significant regional variations in the prevalence of *D. immitis* infection throughout the Americas. Generally, the prevalence is lower in cooler climates, such as

Canada, reaching up to 8.4% in southern Ontario. The prevalence in the United States ranges from 1% to 12% on average, reaching over 40% in highly endemic areas. The prevalence of *D. immitis* infection reaches very high levels in other regions of the Americas, such as the Gulf Coast of Mexico (42%), the Caribbean (63.2%) and Argentina (74%) [20].

The current survey shows a high prevalence (17.56%) in a tropical region of Mexico. The percentage of dogs testing positive via direct ELISA was higher than that of blood samples obtained from domestic canines older than one year in the municipalities of Cuautepec and Acapulco de Juárez, in Guerrero state (a tropical climate), where heartworm prevalence was found to be 15.68% in the municipality of Cuautepec and 7.44% in Acapulco de Juárez [15]. Previous studies have suggested that prevalence varies according to environmental factors, climate and dog population characteristics. For instance, prevalence is generally moderate to high in hot and humid regions of Brazil [21]. In a study conducted in the state of Amazonas in northern Brazil, a heartworm prevalence of 44% was found based on the detection of *D. immitis* DNA [22]. A subsequent study suggested that the average prevalence was higher in the northeast (29.7%) compared to the southeast (26.3%) and southern (13.2%) regions of Brazil [23].

We observed a higher prevalence of positive dogs in the temperate climate (9.56%) compared with reports from the metropolitan area of the city of Puebla, Mexico, where 283 sera were collected from stray dogs and used to detect *D. immitis* antigens using a commercial kit. In that study, *D. immitis* prevalence was 2.12% [24]. However, this differs from a study conducted in eight geographic regions of Mexico, which included 1706 dogs, 37.6% of which were positive for *D. immitis* [13]. In contrast, in Germany, the Netherlands and France, the prevalence of *D. immitis* infection is low, and cases are sporadic, with prevalence decreasing—particularly in former endemic/hyperendemic areas [25]. Similarly, in northern Italy, heartworm prevalence has decreased over the past three decades from >40% to approximately 8% in owned dogs not treated with preventive drugs. Most veterinarians currently surveyed diagnose no more than 5–20 clinical cases per year of *D. immitis* and *D. repens* infections in former hyperendemic areas [26]. Likewise, in the Canary Islands, prevalence decreased from 30% to 19% [27] and it fell from 46% in 1999–2001 to 23% in 2009–2011 in Japan [28].

Results from a serological study in Germany using >80,000 serum samples showed positive *D. immitis* infection in 1.4% of dogs from endemic countries such as Spain, Portugal and Greece [29]. In Greece, the prevalence of heartworm infection ranges from 0.7% to 25%, with the highest values in the country's northern areas [30]. In Romania, canine heartworm prevalence ranges from 3.6% to 14%, depending on the area surveyed, with prevalence up to 42% in stray dogs in the country's southeast. In Turkey, the prevalence ranges from 0–18% [3]. However, these differences could be due to differences in the age group, sex or breed of the dogs sampled, climatic regions, or methods used for diagnosing infection. One of the limitations of the present study was the incomplete inclusion of variables such as age, sex and breeding, indicating that they do not provide complete information about the influence of risk factors on the research outcomes.

The direct diagnosis of heartworm infection may be performed by the detection and identification of circulating microfilariae, while indirect diagnosis is based on the presence of antigens and/or microfilariae in the host. The detection of worms in the heart or subcutaneous tissues either during surgery or after death is conclusive [31,32]. Parasitological laboratory tests (Buffy coat, wet mount and modified Knott's test) and serological tests such as ELISA and immunochromatographic tests for the detection of somatic and female antigens of adult *D. immitis* are available for diagnosis [6,31]. Molecular tests such as polymerase chain reaction (PCR) are currently being recommended to differentiate between microfilaria species [33]. In Brazil, whole blood and serum from 140 dogs were used to evaluate and compare the efficiency and performance of parasitological (capillary blood smear, peripheral and modified Knott's test), serological (rapid immunochromatographic test) and molecular tests (PCR) for the detection of *D. immitis*. The methods evaluated showed high efficiency and reliability, with almost perfect agreement with the PCR considered the gold

standard. The modified Knott's test was the most effective parasitological method, with the highest sensitivity and agreement with PCR [33].

In this study, the sensitivity of the direct ELISA was 100%, and the specificity ranged between 98.2% and 98.8% using a polyclonal antibody to detect serum antigens of *D. immitis* in dogs from the Mexican states of Puebla and Guerrero. A study in Brazil that determined the canine heartworm prevalence by microscopy (19.4%), PCR (15.5%) and ELISA (29.1%) showed that the sensitivity of the ELISA was superior to the other techniques [17]. Similarly, in Romania, a study investigating heartworm infection in 175 dogs found that 21.14% were positive for *D. immitis* antigens while only 16.57% were positive using the modified Knott's technique [34].

Climate change is an important factor that affects the spread of *Dirofilaria* infection, which has led to an increase in mosquito populations, shortening the extrinsic development of infective stages and lengthening the transmission season. Additional factors include the introduction of new invasive and competent mosquito species into the European Union, such as *Aedes albopictus* and *A. koreicus*, the presence of stray dogs with a high prevalence of *Dirofilaria* infection (e.g., 54.8% in Iran and 40.0% in Sofia, Bulgaria) and inadequate heartworm prevention in dogs, especially in new colonisation areas [3]. Several mosquito species, primarily from the genera *Culex*, *Aedes* and *Anopheles*, have been implicated in the transmission of *D. immitis* [35]. In Mexico, *A. taeniorhynchus* and *A. crucians* have been found, consistent with data from the United States [36].

4. Materials and Methods

4.1. Collection of Positive and Negative Samples of D. immitis

Fifty blood samples were obtained from non-stray dogs in a heartworm-endemic area in a tropical region in the State of Guerrero. Blood samples (up to 10 mL per dog) were collected by venipuncture from the cephalic vein into vacutainer tubes with and without EDTA (ethylenediaminetetraacetic acid potassium) anti-coagulant. Samples were individually labelled and stored in vials until analysis.

4.2. Modified Knott's Test

Knott's test was used to detect circulating microfilariae in dogs with a pre-patent infection. One ml of EDTA blood was mixed with 9 mL of 2% formalin in a 12 mL tube and centrifuged for 5 min at 500× *g*. The supernatant was then discarded, and two drops of the sediment were examined by light microscopy (100× or 400× magnification) for the morphological detection and identification of microfilariae using morphometric keys [37]. The positivity or negativity of each sample was confirmed via a qualitative ELISA (SNAP 4Dx Plus, IDEXX Laboratory, Westbrook, ME, USA) using antigens produced by adult female *D. immitis* worms, following the manufacturer's instructions. This test has a sensitivity and specificity of 99.2% and 100%, respectively.

4.3. Study Area and Collection of Samples for Analysis

A total of 335 serum samples were obtained from pet dogs from November 2022 to May 2023 in the municipalities of Quecholac and Tecamachalco in the state of Puebla and from Acapulco and Chilpancingo in the state of Guerrero, which are located in the east-central and southeast regions of Mexico, respectively. The municipalities of Quecholac and Tecamachalco have a surface area of 187.40 km^2 and 180.22 km^2, respectively (Figure 2). The state of Guerrero encompasses the municipalities of Acapulco and Chilpancingo, which cover an area of 1882.6 km^2 and 2180.94 km^2, respectively [38]. The climates of the municipalities located in the states of Puebla and Guerrero are classified as temperate (Cw) and tropical (Aw), respectively, according to the Köppen [39] climatic classification.

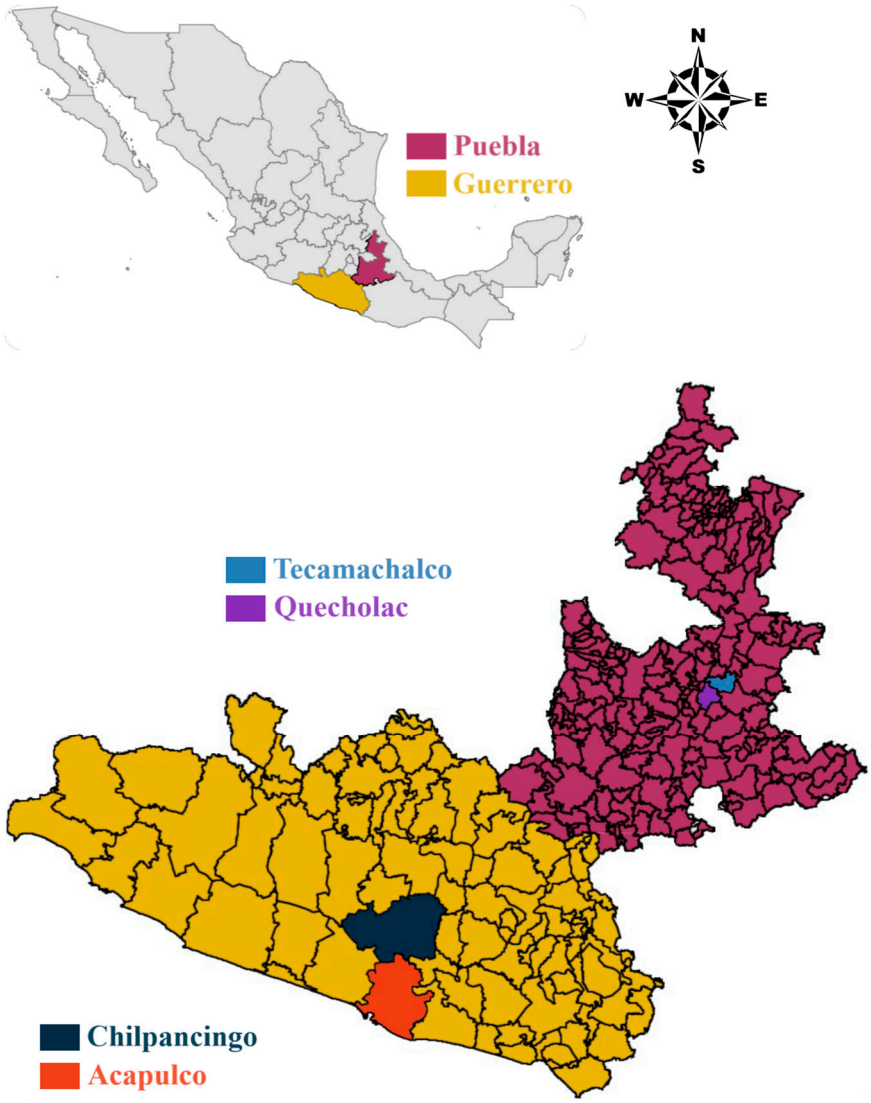

Figure 2. Geographical location of the states of Puebla and Guerrero in a map of Mexico (**upper** map). Location of the four municipalities (**lower** map).

Dog serum was obtained by convenience sampling and based on the willingness of the owners or caregivers to participate in the study. Only dogs over one year of age were selected for study inclusion. There are a lack of data on the total dog population in the participating municipalities, so it is unclear to what extent the participating population is representative of the population in this climate area. The animals were restrained with the owner's help and an experienced veterinarian. Individual blood samples were obtained from each dog during a visit to a clinic for sterilisation surgery, a periodic check-up, annual vaccination or routine health inspection. Blood samples were collected from each animal by cephalic venipuncture into vacutainer tubes without anti-coagulant and kept at room temperature until there was a visible clot reaction. Serum samples were then transported to the laboratory of the Benemérita Universidad Autónoma de Puebla in isothermal containers. The sera obtained after coagulation were centrifuged at 2000× *g* for 10 min, divided into aliquots and stored at −80 °C until use.

4.4. Optimisation of Test Serum Concentration and Secondary Antibodies by Direct ELISA

Dirofilaria immitis nematode antigens in the serum samples were determined using a commercially available polyclonal antibody ELISA (MBS6009306, MyBioSource, Inc., San Diego, CA, USA), optimised by checkerboard titration to determine the optimal concentra-

tions of canine serum and secondary antibody. The absorbance of the assay was measured at 450 nm using an ELISA reader (Biotek ELx800, Winooski, VT, USA).

4.5. Direct ELISA with Test Sera and Positive and Negative Controls

ELISA plates (Costar, Corning, NY, USA) were sensitised with 100 µL of test sera and positive or negative controls per well at a 1:10 dilution with phosphate-buffered saline (PBS). The plates were incubated overnight at 4 °C. The ELISA plate wells were then washed four times with 0.05% PBS-Tween 20. Non-specific sites were blocked with 200 µL of 10% bovine serum albumin (BSA) in PBS-T for 1 h at 37 °C. Following three washes with PBS-T, the plates were incubated with goat anti-*D. immitis* polyclonal antibody (MBS6009306, MyBioSource, Inc., San Diego, CA, USA) conjugated with horseradish peroxidase at a 1:2000 dilution in 1% PBS-BSA and incubated for 1 h at 37 °C. The wells were then washed again as described above, and the reaction colour was developed by adding 100 µL per well of substrate 3,3′,5,5′-Tetramethyl-benzidine (Sigma-Aldrich, St. Louis, MO, USA). The enzyme–substrate reaction was stopped with 50 µL of 4 N H_2SO_4. The absorbance was measured and recorded at 450 nm in an ELISA reader (Biotek ELx800). The results are reported as the mean of the optical density (OD) obtained from duplicate samples.

4.6. Statistical Analysis

The lower limit of positivity (the cut-off value) was established for optimal sensitivity and specificity using the receiver operating characteristic (ROC) curve with a 95% confidence interval (CI) for all serum samples tested via direct ELISA. The ROC curve's cut-off value was determined by selecting the point displaying the highest simultaneous sensitivity and specificity. The accuracy of the area under the curve (AUC) values [40] conformed to the following ranges: uninformative (AUC < 0.5); low accuracy (0.5 < AUC < 0.7); moderate accuracy (0.7 < AUC < 0.9); and high accuracy (0.9 < AUC < 1). A chi-square test was used to assess significant differences in prevalence between different municipalities in the states of Puebla and Guerrero. All data were analysed using SPSS version 25 (IBM Corp., Armonk, NY, USA).

5. Conclusions

The results from this study show that a direct ELISA test based on polyclonal antibodies has a high serodiagnostic value for detecting *D. immitis* serum antigens in dogs from two Mexican states. Our results provide valuable epidemiological data on the present state of *D. immitis* infection in the states of Puebla and Guerrero. Veterinary practitioners play a crucial role in identifying and implementing control methods and preventive measures against the disease in dogs.

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Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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