Canine filariasis in the Amazon: Species diversity and epidemiology of these emergent and neglected zoonoses

Emanuelle Gabriela Gualberto de Argôlo[1]*, Thais Reis[1], Diego Ari Teixeira Fontes[1], Evonnildo Costa Gonçalves[2], Elane Guerreiro Giese[3], Francisco Tiago de Vasconcelos Melo[1], Jeannie Nascimento dos Santos[1], Adriano Penha Furtado[1]

1 Laboratório de Biologia Celular e Helmintologia, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém – Pará – Brazil, **2** Laboratório de Tecnologia Biomolecular, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém – Pará – Brazil, **3** Laboratório de Histologia e Embriologia Animal, Universidade Federal Rural da Amazônia, Instituto de Saúde e Produção Animal, Belém – Pará – Brazil

* emanu_bielle@yahoo.com.br

Abstract

Dirofilaria immitis and *Acanthocheilonema reconditum* are common parasites in dogs but have also been reported parasitizing humans. The differential diagnosis and epidemiological evaluation of these zoonoses are important to the development of efficient public health policies and control strategies. The purpose of this study was to carry out an epidemiological survey by using molecular methods for the specific identification of filarid parasites of domestic dogs in the Marajó mesoregion, State of Pará (PA), Brazil. A total of 418 canine blood samples from Marajó mesoregion (Northern Brazil) were collected, submitted to DNA extraction, polymerase chain reaction (PCR) with "pan filarial" primer, subsequent sequencing and sequence analysis using BLASTn software comparison with previously deposited sequences in GenBank. After that, a phylogenetic analysis by Maximum Parsimony was performed to aid the specific diagnosis. The obtained sequences showed the occurrence of 9 (2.15%) dogs infected with *D. immitis* and 30 (7.18%) by *A. reconditum*, with a confidence interval of 95%, there were no cases of co-infection. We observed that male dogs were more likely to *D. immits* and *A. reconditum* infection. However, age was not significant to both infections. This study reports for the first time the occurrence of *A. reconditum* in the northern region of Brazil and confirmed the presence of *D. immitis* in the Marajó mesoregion.

Introduction

Filariasis can be caused by several onchocercid nematodes that usually have arthropods as intermediate hosts, and can be found parasitizing different species of vertebrates, as definitive hosts [1]. Canine filariases have been reported in several localities around the world, mainly in tropical regions, principally due to the greater concentration and diversity of vectors [2]. Currently, in Brazil, the following filarid species have been found to parasitize canids:

Acanthocheilonema reconditum, *Cercoptifilaria bainae*, *Dipetalonema grassi*, *Dirofilaria immitis* and *Dirofilaria repens* [3].

In these hosts, the adult stages of *A. reconditum*, *C. bainae*, *D. grassi*, and *D. repens* are commonly found in the subcutaneous and/or intramuscular tissue and are considered to be less pathogenic [4]. On the other hand, *D. immitis* adults are located in the pulmonary arteries and right ventricle of the heart, and *D. immitis* is considered the most pathogenic of these filarid species [5].

The occurrence of these infections in Brazil is often related to *D. immitis* [6], and *A. reconditum* reports are limited to one case with a morphological description of circulating microfilariae from dogs in Minas Gerais State (Southeast) [7], epidemiological evaluations with low frequencies in Paraná State (South) [8] and Alagoas (Northeast) [9]. Until now, *A. reconditum* has previously not been reported previously in Brazilian Amazon, specifically in the State of Pará (North).

Zoonotic cases of *D. immitis* and *A. reconditum* occur more frequently in areas with high prevalences of infected canine hosts [4]. However, only *D. immitis* has been previously identified parasitizing humans in Brazil [10–12], but in The Amazon Region, which is considered endemic for canine filariasis and has climatic characteristics that favor the transmission of the parasite, only one human case has been reported [13].

Due to their higher sensitivity and specificity, molecular diagnostic methods, such as nucleotide sequence comparison, are important tools when compared with traditional methods such as blood smear analysis, immunoenzymatic and histochemical tests [14].

The specific diagnosis of these infections in domestic dogs in the Amazon is important because it can be used as a basis to direct health public policies to the proper treatment and control of this infection in both the canine and human populations [15].

Previous works reported dogs infected with *D. immitis* in Marajó mesoregion with high prevalence in some studied areas [16–17]. Thus, the purpose of this study was to carry out an epidemiological study using molecular tools for identification of domestic dogs infected with filarid nematodes in the Marajó mesoregion (Pará State, Brazil), expanding the analyzed area.

Material and methods

Sample collection

Several expeditions to Marajó mesoregion were carried out in the following municipalities: Anajás (00°59'13"S, 49°56'24"W), Chaves (00°09'36"S, 49°59'16"W), Portel (01°56′09″S, 50°49′15″W), São Sebastião da Boa Vista (01°43'04"S, 49°32'27"W) and Soure (00°43′01″S, 48°31′22″W). The expeditions occurred according to the convenience of the health authorities between the years of 2011 to 2014 and were performed by the team of the Cell Biology and Helminthology Laboratory (Institute of Biological Sciences, Federal University of Pará). A total of 418 canine blood samples from domestic dogs living in those areas were collected [Anajás (n = 70), Chaves (n = 28), Portel (n = 141), São Sebastião da Boa Vista (n = 49) and Soure (n = 130)]. The blood was collected via cephalic vein puncture, using disposable sterile needles and stored in tubes with Ethylenediamine tetraacetic acid (EDTA) in a freezer at -20°C, for molecular analysis. The blood samples were collected from dogs with respective owner's agreement, who were informed about the purpose of this research. Blood samples were collected following the order of houses within randomly chosen neighborhoods for sampling. In houses containing more than one dog all animals were sampled except for puppies less than 3 months of age.

Molecular diagnosis

All blood samples were submitted to DNA extraction using phenol-chloroform method [18]. The extracted DNA was subjected to PCR with DIDR F1 and DIDR R1 "pan filarial" primer, to amplify 5.8S–ITS2–28S filarid rDNA regions, according to a protocol established by the authors [14]. The amplicons were visualized after electrophoresis in 1.5% agarose gel stained with SYBR® Green (*Invitrogen*, *California*, *USA*). Canine blood samples were considered positive for filariasis when one or more bands were observed.

The PCR products were cloned into vector p®GEM-T (*Promega*, *Wisconsin*, *USA*) which in turn was introduced into *Escherichia coli* TOP 10 electrocompetent cells (*Invitrogen*, *California*, *USA*) and sequenced with ABI 3130 model automatic DNA analyzer (*Applied Biosystems*, *California*, *USA*), in conjunction with a *BigDye® Terminator v3.1 Cycle Sequencing Kit* (*Applied Biosystems*, *California*, *USA*), according to the manufacturer's specifications.

For a specific diagnosis, the obtained sequences were edited and aligned in the *BioEdit* program [19]. Compared with previously deposited ones in GenBank using BLASTn program [20] additonally a phylogenetic inference was made with all the positive sequences using Maximum Parsimony (MP) method [21] in MEGA 7 program [22] to aid in the identification. The percentage of identical trees in which the associated taxa were grouped (1000 replicates) is shown next to the branches [23]. *Ascaris lumbricoides* (AB571301.1) and *Trichuris trichiura* (KC877992.1) sequences being used as an outgroups. Published sequences from *Dipetalonema reconditum*, now stated as *Acanthocheilonema reconditum* (AF217801.2), *Dipetalonema dracunculoides* (DQ018785.1), *Dirofilaria repens* (AY693808.1) and *Dirofilaria immitis* (EU182330.1) were also used for comparison with the sequences obtained in this work and for the construction of the phylogenetic tree.

All obtained sequences in this work were submitted to GenBank (accession numbers are shown in S1 Appendix).

Epidemiological and statistical analysis

After specific diagnosis, we conducted an epidemiological analysis to investigate parameters that might influence the distribution of these infections. The prevalence of infected dogs was estimated based on specific diagnosis performed through phylogenetic inference. During sampling, information regarding age and sex of all dogs were obtained for association analysis with canine filariasis cases throughout Pearson chi-square test ($X2$), using the BioEstat 5.0 [24] software, with a significance level of 5% ($p<0.05$).

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the "Conselho Nacional de Saúde, Comissão Nacional de Ética em Pesquisa, Fundação Hemopa, Ministério da Saúde" (Permit Number: 0003.0.324.000–10).

Results and discussion

Among the 418 canine blood samples analyzed, 39 (9.33%) were considered positive for canine filariasis based in the presence of visible bands after PCR and electrophoresis. However, of the positive samples, we distinguish 9 samples with bands of approximately 500 bp and another 30 samples with bands of approximately 400 bp. None of the samples presented mixed bands; Rishniw et al. [14] also observed a similar result among their samples, and the authors suggest

that this difference in amplicon size varies according to the filarid species. However, the differences observed after electrophoresis are discrete and insufficient for specific determination.

Thus, all amplicons, were subjected to nucleotide sequencing and BLASTn analysis (S1 Appendix) and 9 (2.15%) have high identity values to *D. immitis* sequences (between 91% to 100%), and the other 30 (7.58%) positive samples presented high identity values to *A. reconditum* (between 80% to 100%). Interestingly, the difference between the band patterns observed in this work was similar to that found by Rishniw et al.[14]. 500 bp for *D. immitis* and 400 bp *A. reconditum*.

To verify the relationships between sequences and to confirm the diversity of the filarid species in dogs from the municipalities of the Marajó mesoregion, a phylogenetic analysis was carried out in all obtained sequences using MP method to generate a cladogram (Fig 1) with two distinct clades:

1. Clade I: formed by a politomy between the 30 sequences of this work and *D. reconditum*, now stated as *A. reconditum* (AF217801.2), with 73% of *Bootstrap*.

2. Clade II: formed by a politomy between the 9 sequences of this work and *D. immitis* (EU182330.1), with 83% of *Bootstrap*.

Furtado et al. [17] performed a filariasis diagnosis based on blood smear analysis, molecular techniques using blood samples and they considered the sequences that formed politomies in the cladogram as the species *D. immitis*. However these authors did not identify *A. reconditum* in their analysis.
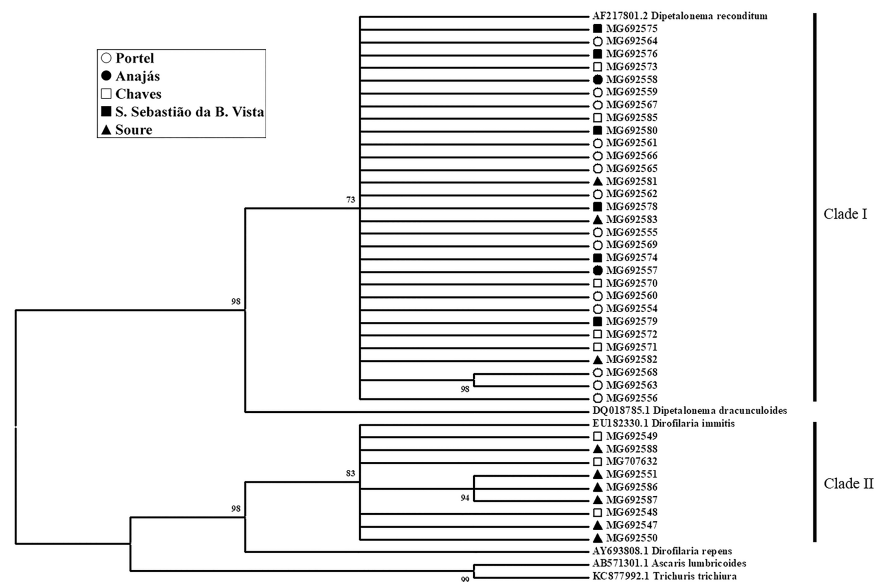
Fig 1. Phylogeny of filarid of the Marajó mesoregion. The cladogram was inferred using the Maximum Parsimony method. It contains a length of 266, the consistency index is 0.614458 and the retention index is 0.872170. The *Bootstrap* is indicated next to the knots of the branches. The sequence of the obtained samples were grouped in two clades: In clade I, 30 sequences of the municipalities Soure, Chaves, Portel, Anajás São Sebastião da Boa Vista formed a politomy with *A. reconditum* (AF217801.2), with *Bootstrap* of 73%. Clade II is composed by 9 sequences of Soure and Chaves municipalities, which form a polytomy with *D. immitis* (EU182330.1) withal 83% of *Bootsrap*. The other filarid sequences remained in isolated branches: *D. dracunculoides* (DQ018785.1) as a brother group of *A. reconditum* (Bootstrap 96%) and *D. repens* (AY693808.1) most related to *D. immitis* (Bootstrap 96%). *A. lumbricoides* (AB571301.1) and *T. trichiura* (KC877992.1) are the outgroup.

This is not the first description of canine filariasis in northern Brazil. Microfilaremic dogs have been reported in several cities, example, in Belém metropolitan region, State of Pará, based on the presence of microfilariae in blood smears [25]; Salvaterra (PA), located in the Marajó mesoregion, using immunoenzymatic diagnosis methods [16]; Porto Velho, State of Rondônia [26] and Lábrea, State of Amazonas [27], using similar methods. However the epidemiological data presented in these studies were associated with *D. immitis* infection, but no specific method for species identification was performed. Thus, it is also possible that cases of misidentification by morphological analysis or cross-reaction in immunoenzymatic essays occurred in those studies. However, an epidemiological study in two municipalities of Marajó Archipelago (Salvaterra and São Sebastião da Boa Vista), using classical and molecular methods for diagnosis, confirmed *D. immitis* parasitizing dogs in these municipalities and highlighted the endemic character of this zoonosis in this area [17].

Our results show for the first time the infection of *D. immitis* in Anajás, São Sebastião da Boa Vista and Portel, with a lower prevalence in Soure and Chaves, additionally, *A. reconditum* was detected in all municipalities studied (Table 1).

Previous studies in Marajó mesoregion, using molecular techniques, PCR and sequencing, have identified only *D. immitis* parasitizing dogs, and the prevalence described in this area differs from that found in this study, since higher percentages in Salvaterra (37.4%) and Sebastião da Boa Vista (6.67%) [17].

The dog gender showed statistically significant association for *D. immitis* and *A. reconditum* infection in Marajó mesoregion, with males having a higher probability of acquiring the infection. On the other hand, the overall age was not significant in both infections. Specifically, in Soure municipality, only age (3 or more years old) was significant for infection by *D. immitis*. In contrast, in Chaves, just males showed to be more susceptible to the infection. The same was observed in Portel, where males were more susceptible to *A. reconditum* infection.

This findings agree with studies performed by Furtado et al. [17] in the same area but differ from what has been reported for other regions [28–29]. Several authors have searched for associations between host gender or age and parasitism by filarids [30–31], and their results have

Table 1. Distribution of filarid infection in domestic dogs by gender and age in domestic dogs from Marajó mesoregion and municipalities: Anajás, Chaves São Sebastião da Boa Vista, Portel and Soure.

Municipality	D. immitis			A. reconditum		
	Positive/tested (%)	p		Positive/tested (%)	p	
		Age	Gender		Age	Sex
Anajás	0/70 (0.0%)	-	-	2/70 (2.86%)	0.6201	0.9966
Chaves	3/28 (10.71%)	0.9248	0.0252*	5/28 (17.86%)	0.4275	0.9602
S. S. Boa Vista	0/49 (0.0%)	-	-	7/49 (14.00%)	0.4349	0.7756
Soure	6/130 (4.62%)	0.0256*	0.0675	4/130 (3.01%)	0.1960	0.9034
Portel	0/141 (0.0%)	-	-	14/141 (9.93%)	0.5784	0.0182*
Gender						
Male	9/418 (2.15%)	0.0166*		23/418 (5.45%)	0.0254*	
Female	0/418 (0.00%)			9/418 (2.13%)		
Age						
0–1 y	0/418 (0.0%)	0.0737		5/418 (1.18%)	0.2246	
>1–3 y	3/418 (0.71%)			13/418 (3.08%)		
>3 y	6/418 (1.44%)			14/418 (3.32%)		
Overall	9/418 (2.15%)			30/418 (7.58%)		

(*) significance level of 5% ($p < 0.05$)

https://doi.org/10.1371/journal.pone.0200419.t001

been shown inconsistent, and no explanations were proposed by those associations. Similar to the results observed in the present work, we did not observe environmental or behavioral characteristics that justified the differences found in gender or age, so we infer that the cause of some associations found herein may be related to the sample number.

However the differences observed here and the factors associated with heartworm transmission in dogs need to be studied in the Amazon Region, since a complex system of interaction between domestic dogs and wild animals can provide differentiated scenarios in the dispersion of infection, as previously described [17].

The data presented in this paper describe for the first time in the Amazon Region the presence of dogs infected with *A. reconditum* (phylogenetic analysis). And this infection seems to be related to gender, but not to age, when analyzing the studied municipalities. None of the work reported the presence of this species of filarid in the Amazon, for this reason we cannot compare our results.

Previous studies conducted in Brazil about *A. reconditum* only described the occurrence, without presenting consistent epidemiological data on this infection. Unfortunately, it was not possible to evaluate if this infection is recent or has been neglected in previous studies in the Amazon, since previous work have only used morphological and immunoenzymatic techniques which may lead to an erroneous species identification. Furthermore, the distribution patterns of *D. immitis* also need to be minutely studied in the Amazon, because, the correct diagnosis and dynamic of infections in the dog population will be important to the development of efficient public health policies, diagnosis and control strategies of zoonotic cases.

Adult stages of *D. immitis* and *A. reconditum* are morphologically different and occupy distinct niches in the definitive host [5]. However, circulatory larval forms (microfilariae) may be confused when observed by light microscopy in routine laboratory diagnosis, even though morphological and biochemical differences have been described among them [32–34]. Although the advantages of the molecular diagnosis of canine filariasis have already been highlighted in other regions around the world [15, 17, 35–36], morphological differentiation is still necessary for taxonomic identification and the detection of *A. reconditum* in the Amazon Region opens up new possibilities for research on morphological and molecular differentiation of this filarid species.

Besides the diversity of canine filarid species in the extensive geographic area of the Amazon, other factors may have influence on the dynamic of those infections in the Amazon, and should be considered, as for example: (1) the high prevalence observed in previous studies; (2) the close association between humans and domestic animals cohabiting both urban and wild environments; and (3) the neglect of these infections by the public health authorities.

In the same manner that sequences of *A. reconditum* was described for the first time in this study, other species not yet recorded, or new species, may be detected in future studies in this area.

Supporting information

S1 Appendix. Tableau with listing of PCR positive samples and their GenBank accession numbers, as well as identities according to BLASTn and accession numbers of the corresponding GenBank sequencesb.
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Evonnildo Costa Gonçalves, Elane Guerreiro Giese, Francisco Tiago de Vasconcelos Melo, Jeannie Nascimento dos Santos, Adriano Penha Furtado.

Data curation: Emanuelle Gabriela Gualberto de Argôlo, Thais Reis, Adriano Penha Furtado.

Formal analysis: Emanuelle Gabriela Gualberto de Argôlo, Elane Guerreiro Giese, Francisco Tiago de Vasconcelos Melo, Jeannie Nascimento dos Santos.

Funding acquisition: Jeannie Nascimento dos Santos, Adriano Penha Furtado.

Investigation: Emanuelle Gabriela Gualberto de Argôlo, Thais Reis, Diego Ari Teixeira Fontes, Adriano Penha Furtado.

Methodology: Emanuelle Gabriela Gualberto de Argôlo, Thais Reis, Diego Ari Teixeira Fontes, Evonnildo Costa Gonçalves, Francisco Tiago de Vasconcelos Melo, Adriano Penha Furtado.

Supervision: Evonnildo Costa Gonçalves.

Writing – original draft: Emanuelle Gabriela Gualberto de Argôlo, Evonnildo Costa Gonçalves, Elane Guerreiro Giese, Jeannie Nascimento dos Santos, Adriano Penha Furtado.

Writing – review & editing: Emanuelle Gabriela Gualberto de Argôlo, Francisco Tiago de Vasconcelos Melo, Jeannie Nascimento dos Santos, Adriano Penha Furtado.

References

1. Anderson R. Nematode parasites of vertebrates their development and transmission. 2nd ed. CABI Publishing; 2000; pp. 482–496.

2. Simón F, Siles-Lucas M, Morchón R, González-Miguel J, Mellado I, Carretón E, et al. Human and animal dirofilariasis: the emergence of a zoonotic mosaic. Clin Microbiol Rev. 2012; 25: 507–544. https://doi.org/10.1128/CMR.00012-12 PMID: 22763636

3. Vicente JJ, Rodrigues HDO, Gomes DC, Pinto RM. Nematóides do Brasil. Parte V: Nematóides de Mamíferos. Rev Bras Zool. 1997; 14: 1–452. https://doi.org/10.1590/S0101-81751997000500001

4. Megat Abd Rani PA, Irwin PJ, Gatne M, Coleman GT, McInnes LM, Traub RJ. A survey of canine filarial diseases of veterinary and public health significance in India. Parasit Vectors. 2010; 3: 30. https://doi.org/10.1186/1756-3305-3-30 PMID: 20377864

5. Irwin PJ, Jefferies R. Arthropod-transmitted diseases of companion animals in Southeast Asia. Trends Parasitol. 2004; 20: 27–34. https://doi.org/10.1016/j.pt.2003.11.004 PMID: 14700587

6. Labarthe N, Guerrero J. Epidemiology of heartworm: what is happening in South America and Mexico? Vet Parasitol. 2005; 133: 149–156. https://doi.org/10.1016/j.vetpar.2005.04.006 PMID: 16198820

7. Gomes LR, Rodrigues RD, de Souza RR, Rodrigues GG, Mundim AV, Barbosa FC. Morphological identification of Acanthocheilonema reconditum in a dog in the municipality of Uberlândia-MG: case report. Veterinária Notícias. 2014; 18: 126–130.

8. Leite LC, Luz E, Cirio SM, Leite MC, Molinari HP, Zadorosnei ACB, Oliveira TMFS, Leite SC. Infection in domestic dogs (Canis lupus familiaris, Linnaeus, 1758) from Guaratuba, Paraná, Brazil by circulating

microfilariae of Acanthocheilonema reconditum (Grassi, 1889). Semin: Ciênc. Agrár. 2012; 33: 1149–1156. https://doi.org/10.5433/1679-0359.2012v33n3p1149

9.   Brito AC, Vila-Nova MC, Martins Rocha DA, Gomes Costa L, Pinheiro de Almeida WA, da Silva Viana L. Prevalence of canine filariasis by Dirofilaria immitis and Dipetalonema reconditum in Maceió, Alagoas State, Brazil. Cad Saude Publica. 2001; 17: 1497–1504.

10. Milanez de Campos JR, Barbas CS, Filomeno LT, Fernandez A, Minamoto H, Filho JV. Human pulmonary dirofilariasis: analysis of 24 cases from São Paulo, Brazil. Chest. 1997; 112: 729–733. https://doi.org/10.1378/chest.112.3.729 PMID: 9315807

11. Cavallazzi RS, Cavallazzi AC, Souza IV, Deus Cardoso JJ. Dirofilariose pulmonar humana: relato de sete casos. J Pneumologia. 2002; 28: 100–102. https://doi.org/10.1590/S0102-35862002000200007

12. Rodrigues-Silva R, Guerra RJ, de Almeida FB, Machado-Silva JR, de Paiva DD. Human pulmonary dirofilariasis at Rio de Janeiro, Brazil: a case report. Rev Soc Bras Med Trop. 2004; 37: 56–59. https://doi.org/10.1590/S0037-86822004000100015 PMID: 15042186

13. Otranto D, Diniz DG, Dantas-Torres F, Casiraghi M, de Almeida INF, de Almeida LNF, et al. Human intraocular filariasis caused by Dirofilaria sp. nematode, Brazil. Emerg Infect Dis. 2011; 17: 863–866. https://doi.org/10.3201/eid1705.100916 PMID: 21529396

14. Rishniw M, Barr SC, Simpson KW, Frongillo MF, Franz M, Alpizar JL. Discrimination between six species of canine microfilariae by a single polymerase chain reaction. Vet Parasitol. 2006; 135: 303–314. https://doi.org/10.1016/j.vetpar.2005.10.013 PMID: 16289566

15. Casiraghi M, Bazzocchi C, Mortarino M, Ottina E, Genchi C. A simple molecular method for discriminating common filarial nematodes of dogs (Canis familiaris). Vet Parasitol. 2006; 141: 368–372. https://doi.org/10.1016/j.vetpar.2006.06.006 PMID: 16857318

16. Garcez LM, de Souza NF, Mota EF, Dickson LA, Abreu WU, Cavalcanti Vde F, et al. Focus of canine heartworm disease in Marajó Island, North of Brazil: A risk factor for human health. Rev Soc Bras Med Trop. 2006; 39: 333–336. https://doi.org/10.1590/S0037-86822006000400003 PMID: 17119746

17. Furtado AP, Do Carmo ES, Giese EG, Vallinoto AC, Lanfredi RM, Santos JN. Detection of dog filariasis in Marajo Island, Brazil by classical and molecular methods. Parasitol Res. 2009; 105: 1509–1515. https://doi.org/10.1007/s00436-009-1584-9 PMID: 19693539

18. Sambrook J, Russell DW. Molecular cloning: A laboratory manual, Vol. 3. New York: Cold Spring Harbor Laboratory Press; 2001.

19. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp. 1999; 41: 95–98.

20. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol. 1990; 215: 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2 PMID: 2231712

21. Nei M, Kumar S. Molecular Evolution and Phylogenetics, New YorK: Oxford University Press; 2000.

22. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016; 33: 1870–1874. https://doi.org/10.1093/molbev/msw054 PMID: 27004904

23. Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. Evolution. 1985; 39: 783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x PMID: 28561359

24. Ayres M, Ayres Junior M, Ayres DL, Santos AS. Bioestat 5.0 –Aplicações estatísticas nas áreas das ciências biomédicas. Belém: ONG Mamiraua. 2007.

25. Souza NF, Benigno RNM, Figueiredo M, Salim SK, Silva D, Goncalves R, et al. Prevalência de Dirofilaria immitis em cães no município de Belém—PA, com base na microfilaremia. Rev Bras Parasitol Vet. 1997; 6: 83–86.

26. Ogawa GM, Da Cruz EN, Cunha PN, Camargo LM. Canine heartworm disease in Porto Velho: first record, distribution map and occurrence of positive mosquitoes. Rev Bras Parasitol Vet. 2013; 22: 559–564. https://doi.org/10.1590/S1984-29612013000400017 PMID: 24473882

27. Soares HS, Camargo LM, Gennari SM, Labruna MB. Survey of canine tick-borne diseases in Lábrea, Brazilian Amazon: 'accidental' findings of Dirofilaria immitis infection. Rev Bras Parasitol Vet. 2014; 23: 473–480. https://doi.org/10.1590/S1984-29612014093 PMID: 25517525

28. Nzabanita E, Priester W, Ferver T. Distribution of canine filariasis. Calif Vet. 1982; 36: 24–28.

29. Oge H, Doğanay A, Oge S, Yildirim A. Prevalence and distribution of Dirofilaria immitis in domestic dogs from Ankara and vicinity in Turkey. Dtsch Tierarztl Wochenschr. 2003; 110: 69–72. PMID: 12666502

30. Montoya JA, Morales M, Juste MC, Bañares A, Simon F, Genchi C. Seroprevalence of canine heartworm disease (Dirofilaria immitis) on Tenerife Island: an epidemiological update. Parasitol Res. 2006; 100: 103–105. https://doi.org/10.1007/s00436-006-0257-1 PMID: 16821033

31. Boonyapakorn C, Srikitjakarn L, Morakote N, Hoerchner F. The epidemiology of Dirofilaria immitis infection in outpatient dogs at Chiang Mai University Small Animal Hospital, Thailand. Southeast Asian J Trop Med Public Health. 2008; 39: 33–38. PMID: 18567441

32. Anderson RC, Bain O. Keys to Genera of the Order Spirurida. In: Anderson RC, Chabaud AG, Willmott S, editors. Keys to the Nematode Parasites of Vertebrates. England: C.A.B. Commonwealth Agricultural Bureaux; 1976; pp. 59–116.

33. Song KH, Hayasaki M, Cho KW, Lee SE, Kim DH. Cross-reactivity between sera from dogs experimentally infected with Dirofilaria immitis and crude extract of Toxocara canis. Korean J Parasitol. 2002; 40: 195–198. https://doi.org/10.3347/kjp.2002.40.4.195 PMID: 12509105

34. Magnis J, Lorentz S, Guardone L, Grimm F, Magi M, Naucke TJ. Morphometric analyses of canine blood microfilariae isolated by the Knott's test enables Dirofilaria immitis and D. repens species-specific and Acanthocheilonema (syn. Dipetalonema) genus-specific diagnosis. Parasit Vectors. 2013; 48: 25.

35. Xie H, Bain O, Williams SA. Molecular phylogenetic studies on filarial parasites based on 5S ribosomal spacer sequences. Parasite. 1994; 1: 141–151. https://doi.org/10.1051/parasite/1994012141 PMID: 9140481

36. Nuchprayoon S, Junpee A, Poovorawan Y, Scott AL. Detection and differentiation of filarial parasites by universal primers and polymerase chain reaction-restriction fragment length polymorphism analysis. Am J Trop Med Hyg. 2005; 73: 895–900. PMID: 16282300