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ORIGINAL PAPER

PCR detection of *Dirofilaria immitis* in *Aedes aegypti* and *Culex pipiens* from urban temperate Argentina

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Abstract Dirofilariasis, a mosquito-borne disease of dogs caused by the nematode *Dirofilaria immitis* (Leidy; Spirurida: Onchocercidae), has now become a growing zoonotic concern. Based on direct microscopical observation, *Aedes aegypti* (L.) and *Culex pipiens* L. (Diptera: Culicidae) have been previously incriminated as potential vectors of *D. immitis* in urban temperate Argentina. In this study, an effort was made to provide evidence for this assumption by screening of mosquitoes for *D. immitis* infection using a polymerase chain reaction (PCR) assay. PCR primers were developed to specifically amplify the *D. immitis*-16S rRNA gene and to reliably detect 100th of the genomic equivalent (10 pg) of the infective third-stage larvae in mosquito pools of up to 30 individuals. Collection of mosquitoes was performed between September 2007 and April 2008 in premises known to be inhabited by *D. immitis*-infected dogs in Greater Buenos Aires. The final collection comprised 453 specimens belonging to 11 mosquito species of the genera *Aedes*, *Culex*, *Ochlerotatus*, and *Psorophora*. PCR assays were performed on 82 pools ($n \leq 20$) of heads and abdomens separately, as this allows differentiating infective and non-infective stages of the parasite, respectively. Identification of the non-infective stage of *D. immitis* in *A. aegypti* and *C. pipiens* provided additional strong support of transmission of the parasite by these species. To our knowledge, this was the first PCR screening for *D. immitis*-infected mosquitoes in South America.

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Introduction

The dog heartworm, *Dirofilaria immitis* (Leidy; Spirurida: Onchocercidae), is considered a veterinary concern in most warm regions of the word. The nematode, transmitted by mosquitoes (Diptera: Culicidae), infects also domestic cats and a wide range of wild animals (Anderson 2000), and is currently included amongst the canine diseases of zoonotic concern (Dantas-Torres 2008; Otranto et al. 2009). During the last century, important aspects of dirofilariasis have been elucidated, but there are many unanswered questions

in particular concerning the species that can act as vectors in the world (Cancrini and Gabrielli 2007). Since the first report of a mosquito infected with *D. immitis* in 1901, more than 60 mosquito species have been incriminated as potential vectors (Ludlam et al. 1970). Traditionally, the identification of the filarial larval stages in the mosquito is based on morphological criteria by microscopical diagnostics, which is considered to be laborious, of low sensitivity, and can lead to misdiagnosis (Nuchprayoon et al. 2005; Plichart et al. 2006; Scoles and Kambhampati 1995; Watts et al. 1999). The development of molecular methods to identify filarial larvae has allowed a more accurate identification of their mosquito vectors during the last decade (McCall et al. 2008).

In South America, canine dirofilariosis has already been reported in the year 1875 (Labarthe and Guerrero 2005), but information about the transmitting mosquito species is still scarce. In Brazil, the occurrence of *D. immitis* has been recorded in natural populations of *Aedes taeniorhynchus* (Wiedmann), *Ochlerotatus scapularis* (Rondani), *Culex quinquefasciatus* Say, *Culex declarator* Dyar and Knab, *Culex saltanensis* Dyar, and *Wyeomyia bourrouli* Lutz (Ahid and Lourenço de Oliveira 1999; Labarthe et al. 1998; Lourenço de Oliveira and Deane 1995). In contrast, only *Aedes aegypti* (L.) and *Culex pipiens* L. have been found to be infected with this parasitic nematode in Argentina so far (Vezzani et al. 2006). Furthermore, reports on *D. immitis* vectors do not exist in other countries of South America and screening of the pathogen in potential vector mosquitoes using polymerase chain reaction (PCR)-based assays has to our knowledge not been carried out in the continent.

In a previous survey in temperate Argentina, only three infected specimens have been detected in 2,380 mosquitoes by direct microscopical observation (Vezzani et al. 2006). To increase the chance of detection of helminth-infected mosquitoes, the study at hand was conducted with mosquitoes that had been captured exclusively within premises inhabited by infected dogs and using highly sensitive and specific molecular diagnostics (PCR) for parasite detection.

Materials and methods

Study area and mosquito sampling

Greater Buenos Aires has a climate temperate humid with four seasons, mean annual rainfall of 1,076 mm, and mean annual temperature of 17.4°C (IGM 1998). Mosquito collections were performed in eight private premises located in the municipalities of Avellaneda, Lomas de Zamora, Quilmes, and Almirante Brown. These premises were chosen due to the presence of inhabiting microfilaremic dogs with serologic confirmation of *D. immitis* infection at the beginning of the study. Between September 2007 and April 2008 a total of 33 mosquito surveys were done using oral aspirators and sweep nets by disturbing vegetation and nearby places. The collection included mosquitoes in a host-seeking behavior and those resting on natural or man-made structures. Collections of resting mosquitoes usually provide more representative samples of the population as a whole than most other methods, with the additional advantage of catching blood-fed and gravid females (Silver 2008).

The mosquitoes were transported alive to the laboratory and maintained at 5°C until taxonomic identification using the key of Buenos Aires mosquitoes (Rossi et al. 2002). The specimens were pooled up to 20 individuals according to species, collection date, and site. Heads and abdomens were maintained as separate pools to differentiate samples positive for infective and non-infective stages of the parasite, respectively. As a negative control, adult female mosquitoes from a laboratory rearing were used. All pools were stored in 70% ethanol at −20°C until isolation of genomic DNA was carried out.

DNA isolation

Genomic DNA was extracted from blood of a *D. immitis*-infected dog using the DNeasy Blood and Tissue Kit as recommended by the manufacturer (Qiagen, Hilden, Germany). Mosquito and microfilaria-mocked mosquito samples were ground in a 1.5 ml tube using a sterile disposable microtube pestle, respectively. The same genomic DNA isolation Kit as above was used but the workflow for insect specimens was carried out as recommended by the manufacturer. Genomic DNA was finally resolved in 100-µl TE buffer and stored at −20°C.

PCR analysis and sequencing

Amplifications were carried out in a PCR buffer (20 mM Tris–HCl, pH 8.4, and 50 mM KCl) containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1 µM of forward and reverse primer, and 0.75 U of Taq polymerase. Water and 10 µl of the genomic DNA sample were added to make up a final reaction volume of 25 µl. Thermal cycle reactions were set to a first denaturing step of 95°C for 10 min, followed by 35 cycles each composed of 95°C for 60 s, 55°C for 45 s, 72°C for 60 s, and eventually terminated by cooling to 4°C until amplicons were being processed. Amplification products were resolved on ethidium bromide stained 1% agarose gels by electrophoresis in Tris–acetate EDTA buffer. A 1 kb DNA Ladder (Promega, Madison, WI) was run in parallel to allow size estimation of observed

bands. For verification of the identity of observed with expected DNA amplicons, obtained bands were eluted from the gel using the illustra GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare Waukesha, WI) as described by the manufacturer. The eluted DNA was subjected to direct sequencing of both strands using the respective PCR primers as forward and reverse sequencing primers.

Results

Search in the GenBank database for genes that might be suitable targets for molecular diagnostics of *D. immitis* resulted in the identification of a ribosomal 16S rRNA partial gene (Watts et al. 1999). Forward Di_16S_RNA-F: AGCTCGTAGTTGGATCTGCAT and reverse amplification primer: DI_16S_RNA-R: CGTCAAGGCGTATTTACCG were designed to amplify a 453 bp DNA product. In preliminary amplification tests the designed PCR primers faithfully amplified a fragment of the expected size from genomic DNA of blood that had been isolated from a *D. immitis*-infected dog (Fig. 1). The identity of the observed with the expected 453 bp amplicon was verified by sequencing (nucleotide sequence data is available under accession number: HM124350). Importantly, DNA that had been isolated from pools of 20 non-infected mosquito heads or abdomens did not result in amplification of a product. To the best of our knowledge, from Argentina there are no published records of other filarial species in mosquitoes and dogs besides *D. immitis*, with which our primers could potentially cross-react. Accordingly, genomic DNA of other parasitic filarioidea that may infect mosquitoes and dogs is not available. Nevertheless, we carried out a specificity check of our primers against all nucleotide entries of filarioidea deposited in the GenBank database (69 species) using Primer-BLAST (Rozen and Skaletsky 2000). No cross-reactivity with any sequence entry of parasitic filarioidea could be observed underlining the specificity of the developed primers.

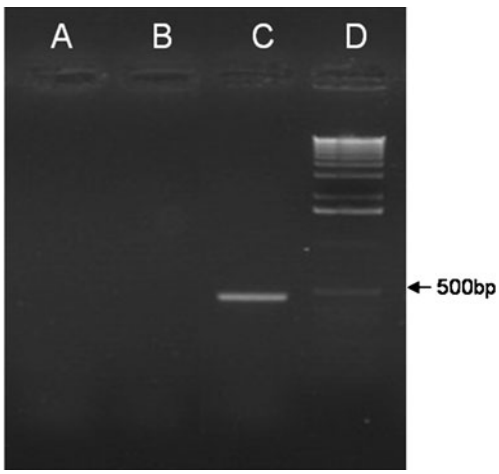

Fig. 1 PCR amplification of a *Dirofilaria immitis* 16S rRNA gene fragment. Agarose gel electrophoresis was carried out with PCR products generated by amplification of genomic DNA extracted from uninfected mosquitoes (*A*) and from blood of a *D. immitis*-infected dog (*C*). A PCR-negative control was run in (*B*) and a 1 kb DNA ladder was used as a marker for size estimation of DNA fragments in (*D*)

To establish the detection limit of this PCR test, a quantity of DNA reported to be present in the infective stage L_3 (10 pg) was subjected to amplification (Chanteau et al. 1994). This reliably resulted in observation of the expected band and amplification was still observed after applying the hundredth part (0.1 pg) of this DNA quantity (data not shown). Thus, the diagnostic primers used in this study show a similar sensitivity as the primers targeting the same 16S RNA gene reported by Watts et al. (1999).

Pools of either 20 or 30 mosquito heads or abdomens were each spiked with an amount of genomic DNA corresponding to that contained by a single microfilaria to assess a possible interference of mosquito DNA with *D. immitis* detection. Amplification of the genomic DNA isolated from each of these pools resulted always in the appearance of a band of the expected size demonstrating that the assay is robust and perfectly applicable for screening of *D. immitis*-infected mosquitoes in pools comprising less than 30 mosquitoes.

A total of 453 female individuals of 11 mosquito species were collected and separated in 82 pools ($n \leq 20$) of heads and abdomens, respectively (Table 1). Amplification of a band of the expected size was exclusively observed in two pools of abdomens of *A. aegypti* and one of *C. pipiens* demonstrating the presence of the non-infective *D. immitis* stage in these mosquito species. The identity of observed bands was confirmed by direct sequencing (nucleotide sequence data are available under accession numbers: HM124347, HM124348, and HM124349). *D. immitis*-infected pools corresponded to mosquitoes collected at the end of summer in the municipalities of Quilmes, Avellaneda, and Lomas de Zamora (Fig. 2).

Discussion

Infection prevalences in natural mosquito populations have been consistently reported to be lower than 4% in studies carried out in countries so distant like Brazil (Labarthe et al. 1998), Taiwan (Lai et al. 2001), and Italy (Cancrini et al. 2006). In the present study, three infected pools of abdomens out of 82 tested were detected, representing a minimum infection prevalence of 0.7% (3/453) assuming the presence of one infected mosquito per pool. Most remarkably, this

Table 1 Number of female individuals collected for each mosquito species in Greater Buenos Aires between September 2007 and April 2008, and number of pools tested for presence of *Dirofilaria immitis* by PCR

Mosquito species	No. of individuals	No. of pools
Ochlerotatus albifasciatus	148	20
Culex pipiens	147	19
Aedes aegypti	90	22
Culex apicinus	41	11
Psorophora albigenu	9	1
Culex maxi	7	3
Culex chidesteri	3	2
Culex eduardoi	3	1
Culex dolosus	2	1
Culex bidens	2	1
Ochlerotatus crinifer	1	1

value is seven times higher than that found by microscopic examination in the same study area (Vezzani et al. 2006). This difference is probably due to the sensitivity of the diagnostic technique and the mosquito source. The present findings support the idea that PCR methods are considerably more sensitive than microscopical investigations, besides the additional advantage of exact parasite species identification.

Up to now, there is no record of the infective stage (L_3) of the dog heartworm in any mosquito species in Argentina. In this sense, the current molecular survey validates the potential role of *A. aegypti* and *C. pipiens–quinquefasciatus* complex as *D. immitis* vectors, as was previously suggested by microscopic observation by Vezzani et al. (2006). In this context, it is noteworthy that in Brazilian strains of both mosquito species the parasite reached the infective stage in experimental infections (Ahid et al. 2000; Brito et al. 1999; Macedo et al. 1998; Serrão et al. 2001). Furthermore, *C. quinquefasciatus* was found to harbor the infected stage in field caught specimens in Rio de Janeiro (Labarthe et al. 1998) and São Luis (Ahid and Lourenço de Oliveira 1999). Apart from these South American investigations, natural transmission of *D. immitis* by both mosquito species has also be demonstrated in other geographic regions (Cancrini and Gabrielli 2007; Hendrix et al. 1986). Considering the domestic nature of both container-breeding mosquito species, the control of breeding sites in the peridomicile of dog owners would be most likely effective in diminishing local transmission. Of the other mosquito species collected, none was previously found to be naturally infected, and under experimental conditions the parasite has reached the infective stage only in *Ochlerotatus albifasciatus* (Bacigalupo 1945).

According to the data of the current and previous surveys (Vezzani et al. 2006), *A. aegypti* and *C. pipiens* are two of the most frequent mosquito species collected in premises of urban temperate Argentina. The collection dates of the infected mosquitoes perfectly match with the hypothetical period of highest transmission risk of heartworm (Vezzani and Carbajo 2006) and also with the highest adult productivity of both species in Buenos Aires (Vezzani and Albicócco 2009). The great abundance of these mosquito species in domestic environments at the end of summer together with their host-feeding patterns, highlight their potential role in a possible zoonotic transmission cycle. The relative low number of human dirofilariasis cases reported in Argentina may be due to the asymptomatic nature of infection and diagnostic problems, as has been suggested for other countries (Theis 2005).

To our knowledge, the present study was the first PCR screening for *D. immitis*-infected mosquitoes in South America. Our findings provided additional evidence of

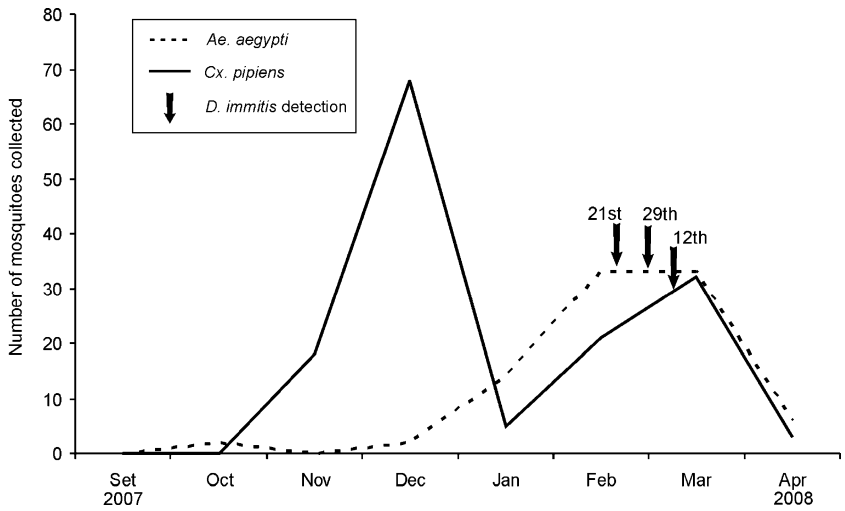

Fig. 2 Monthly patterns of abundance of *Aedes aegypti* and *Culex pipiens* adults, and dates of detection of *Dirofilaria immitis*-infected mosquitoes in Greater Buenos Aires; September 2007–April 2008

parasite transmission by *A. aegypti* and *C. pipiens* supporting the available *D. immitis*-transmission model based on thermal regime and presumed vectors.

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