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Evaluation of Larval Development of *Dirofilaria immitis* in Different Populations of *Aedes aegypti* and *Aedes albopictus*

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ABSTRACT

Dirofilaria immitis is an important nematode parasite commonly known as heartworm. This filarioid is transmitted by culicid vectors and primarily affects dogs, but other animals may also become infected, such as wild carnivores, cats and humans. The aim of the present study was to assess the development of *D. immitis* larvae in different culicid populations under laboratory conditions. Adult females of populations of *Aedes aegypti* from the city of Recife (P1), the city of Campinas (P2) and the Rockefeller strain from the Centers for Disease Control (P3) and one population of *Aedes albopictus* from Recife (P4) were fed for two hours with infected dog blood containing 2,000 microfilariae/ml of *D. immitis*. After artificial feeding, the specimens were maintained under controlled conditions. Ten females from each population were dissected daily over 14 days. The infection ratio and vector efficiency index were calculated and *D. immitis* development from L_1 to L_3 was assessed. The larvae in P1, P2, P3 and P4 reached the third stage in 11, 10, 14 and 9 days, respectively. The vector efficiency index was 53.8%, 20.0%, 7.4% and 25.2% in P1, P2, P3 and P4, respectively. The findings demonstrate that *D. immitis* larvae develop in all culicid populations studied herein. Based on mosquito mortality, development time and VEI the *A. albopictus* population from Recife (P4) demonstrated the best performance as vector. This is the second report of *D. immitis* development in *A. albopictus* from Brazil. The present data reinforce the role of this species as vector of *D. immitis* in an area where greater importance has long been given to *A. aegypti*.

Keywords: Culicid; Vector; Heartworm; Dog; Experimental Infection

1. Introduction

Dirofilariasis is an important parasitic disease caused by the nematode *Dirofilaria immitis*, also known as heartworm [1]. This filarioid primarily affects dogs, but infection has also been reported in cats [2,3], wild carnivores [4,5] and humans [6]. In this later host, infection is characterized by the presence of pulmonary nodules (the so-called "coin lesion") and no apparent symptoms [7].

Transmission occurs through mosquito vectors, which are the most important vectors that belong to the genera *Culex*, *Aedes* and *Anopheles* [8,9]. Briefly, first stage larvae (L_1) are taken up by blood-sucking female mosquitoes and develop through to the infective final larvae stage (L_3) in approximately 14 days [10]. The vector competence of a culicid population is the primary factor for *D. immitis* transmission in an endemic area. However, characteristics such as the immune reaction, environmental conditions (e.g., temperature and humidity) and genetic traits among *D. immitis* populations may be factors which affect the vector capacity of culicids [11].

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In Brazil, studies on *D. immitis* vectors are scarce. In the southeastern region of the country, *Aedes scapularis* and *Aedes taeniorhynchus* have been shown to be the main vectors, while *Culex quinquefasciatus* seems to have secondary importance [2]. Also in this region (state of Rio de Janeiro), an experimental study demonstrated *Aedes aegypti* as a potential vector of *D. immitis* [12]. In the northeastern region *Cx. quinquefasciatus* and *A. taeniorhynchus* have been found naturally infected [13], but little is known regarding the epidemiological importance of these species, especially the latter. Unfortunately, the vector competence of two species well established throughout Brazil (*A. aegypti* and *A. albopictus*), has been poorly studied.

Therefore, the aim of the present study was to assess the development of *D. immitis* larvae in three populations of *A. aegypti* and one of *A. albopictus* from different geographic areas under laboratory conditions.

2. Material and Methods

2.1. *D. immitis* Microfilariae

Blood containing microfilariae was obtained from a dog positive for *D. immitis* in the microscopic analysis (see below). The dog was a three-year-old male that lived in the metropolitan region of Recife (7˚45'0"S and 34˚51'0"W), state of the Pernambuco, Brazil.

2.2. Microscopic Diagnosis (Modified Knott Test)

The microscopic analysis was performed using the modified Knott test [14]. Briefly, 1 ml EDTA blood was mixed with 9 ml of distilled water and centrifuged for 5 min at 300 × g. The supernatant was discarded, leaving 1 ml of solution to which two drops of methylene blue solution were added. The sediment was transferred to glass slides, covered with coverslip and examined under a light microscope at different magnifications. All filarioid larvae found were morphologically identified as *D. immitis*.

2.3. Mosquito Populations and Experimental Infection

Four different mosquito populations were used: *A. aegypti* from the city of Recife, Brazil (P1), *A. aegypti* from the city of Campinas, Brazil (P2), *A. aegypti* Rockfeller strain from the Centers for Disease Control in the USA (P3) and *A. albopictus* from Recife (P4).

A total of 4,800 female mosquitoes (3,600 for the test populations and 1,200 for the control) aged from three to seven days were used [15]. The artificial blood meal was performed as previously described [10]. The test mosquitoes were fed for two hours with the infected blood, which was previously assessed as containing 2,000 microfilariae/ml. Non-infected blood was offered to the control group. After feeding, the mosquitoes were maintained under controlled temperature (28˚C ± 2˚C) and relative humidity (>70%).

2.4. Mosquito Dissection and Microscopic Examination

For the detection of *D. immitis* larvae and developmental stages, ten mosquitoes from each population were dissected daily. On Day 14, all remaining living mosquitoes were also dissected. The mosquitoes were fixed on slides containing a drop of 0.9% physiological saline solution, dissected with a sterile scalpel and immediately examined under a light microscope (Olympus BX41 TF) at different magnifications. All stages of *D. immitis* larvae were morphologically identified [16].

2.5. Data Analysis

The infection ratio (IR) [17] and vector efficiency index (VEI) were calculated [18]. Linear regression was used to determine the influence of the ingestion of *D. immitis* microfilariae on mosquito mortality. Analysis of variance was used to compare larval development up to the infectious stage in the female mosquitoes as well as mortality in the different populations. The BioStat program (version 2.0) was employed for the statistical analysis [19].

3. Results

After feeding, the mosquitoes exhibited a similar rate of engorgement: 97.6%, 91.5%, 97.6% and 93.3% in P1, P2, P3 and P4, respectively, and 96.6% in the control group. **Table 1** displays the mean ingestion of microfilariae in each population. Microfilaria ingestion was statistically similar among P1, P3 and P4 ($P > 0.05$), whereas P2 ingested a significantly smaller number of microfilariae ($P < 0.01$).

Throughout the study period, mosquito mortality was greater in P1 than P2, P3 and P4 ($P < 0.01$). However, this higher mortality rate was not influenced by the number of microfilariae ingested ($F = 0.6899$; $P > 0.05$). Two peaks in mosquito mortality were found in all populations, one on the second day post-infection and one after the detection of L_3. At the end of the study, overall mortality in the control group was 3.4%.

The infection rate among the populations ranged from 11.6% to 17.5% (**Table 2**). This parameter was not influenced by the proportion of engorged females ($F = 12.8754$; $P > 0.05$), but was affected by the number of microfilariae ingested ($F = 141.3808$; $P < 0.01$).

D. immitis larval development from L_1 to L_3 occurred in all infected populations. In P1, L_1 was found on Day 3 post-infection, L_2 on Day 7 and a low number of L_3 ($n = 7$) were retrieved on Day 11. In P2 and P3, L_1 on Day 6 post-infection, L_2 was found on Day 8 and L_3 was found

Table 1. Mean number of microfilariae ingested, rate of recovery of L_3 and VEI in mosquito females of different populations (*A. aegypti* Recife—P1, *A. aegypti* Campinas—P2, *A. aegypti* lineage Rockfeller—P3 and *A. albopictus*—P4). Specimens were fed with dog blood containing 2,000 microfilaria/ml of *D. immitis*.

Pop.	Mean of L_1 ingested ± SD*	Mean of L_3 ± SD**	VEI (%)
P1	13 ± 10.4^{a}	7.0 ± 0^{B}	53.8
P2	5 ± 4^{b}	1 ± 0^{A}	20
P3	13.4 ± 7.7^{a}	1 ± 0^{A}	7.4
P4	11.3 ± 9.4^{a}	2.8 ± 2^{C}	25.2

*Mean number of microfilariae ingested after blood meal; **Mean number of (L_3) recovery 14 days post-infection; different letters in the same column indicate statistical difference.

Table 2. Total number of dissected and infected mosquito females of different populations (*A. aegypti* Recife—P1, *A. aegypti* Campinas—P2, *A. aegypti* lineage Rockfeller—P3 and *A. albopictus*—P4). Specimens were fed with dog blood containing 2,000 microfilaria/ml of *D. immitis*.

Pop.	Dissected females	Infected females n (%)
P1	849	149 (17.5%)
P2	821	95 (11.5%)
P3	878	149 (16.9%)
P4	838	131(15.6%)

on Days 10 and 14, respectively. Melanized larvae and few larvae remaining as L_1 were detected at the end of the study in all populations. *D. immitis* larvae exhibited a comparatively short development time when infecting *A. albopictus* (P4), with larvae reaching L_1, L_2 and L_3 at Days 2, 5 and 9, respectively.

The number of L_3 obtained 14 days post-infection (**Table 1**) was not influenced by the number of microfilariae ingested ($F = 0.0415$; $P > 0.05$). The VEI ranged from 7.4% to 53.8% in the different populations (**Table 1**).

4. Discussion

The present study provides evidence that *D. immitis* larvae can develop efficiently in all culicid populations studied herein. Based on mortality, development time and VEI the *A. albopictus* population (P4) demonstrated the best performance as vector of this nematode.

The mean ingestion of microfilariae was similar among P1, P3 and P4, whereas P2 ingested a significantly smaller number. From a biological standpoint, microfilaria ingestion is an important limiting factor related to the vector competence of a mosquito population. However, the mechanisms that impair the ingestion of *D. immitis* microfilariae and lead to mosquito infection remain unclear. It has been shown that some mosquito populations have efficient defense mechanisms against species of *Dirofilaria* soon after ingestion [20]. Probably, when microfilariae reach the Malpighian tubule, intracellular development is blocked by defense mechanisms activated by the host, resulting in larval death and lysis [20].

A greater mortality rate was found among the mosquitoes in P1. Interestingly, this parameter was not influenced by the number of microfilariae ingested ($F = 0.6899$; $P > 0.05$). Both mortality peaks occurred during critical post-infection times: first during the penetration of L_1 into the Malpighian tubule cells (between Days 1 and 2 post-infection) and soon after the detection of L_3. In both events, Malpighian tubule cells were destroyed, with the magnitude of the destruction depending on the quantity of larvae, causing death in P1. As previous states [21] an efficient mosquito vector should survive independently of the number of ingested microfilariae thus, P1 may exhibit a comparatively low degree of vector competence.

D. immitis development was observed in all populations studied herein, with the maximum VEI (53.8%) found for *A. aegypti* from Recife (P1). However, this population may not be considered an efficient vector due to the high mortality rate observed (70.7%). The minimal differences in development time found among the populations of *A. aegypti* were expected, since the same nematode strain was used for all populations. Previous studies have been reported different development times for this filarioid when different strains of nematode and mosquitoes are used [16,22].

Among the four populations studied, one merits particular attention, *A. albopictus* from Recife (P4) exhibited low mortality and a considerable VEI (25.2%). Furthermore, larvae reached the third infective stage in only nine days. *A. albopictus* is reported to be the primary potential vector of *D. immitis* in Italy [23]. The importance of this mosquito as a vector for *D. immitis* in Brazil has been assessed in a single study [12]. Nonetheless, its vector capacity has interesting epidemiological implications. *A. albopictus* is considered a secondary vector of human arboviruses due to its preferential feeding on animals rather than humans, unlike the highly anthropophilic behavior of *A. aegypti* [24]. Based on feeding preference *A. albopictus* may have a major veterinary importance for *D. immitis* transmission among animals, which should be studied better. It is important stress that the *A. albopictus* population evaluated herein proved to be susceptible to infection and allowed *D. immitis* larvae development. However, further studies evaluating the vector capacity of other populations of this species in different regions of Brazil should be carried out.

5. Conclusion

In conclusion, *D. immitis* larvae developed in all mosquito populations studied. Based on factors as mortality, development time and VEI, the *A. albopictus* population from Recife demonstrated the best performance as vector. This study is the second description of the development of *D. immitis* in *A. albopictus* in Brazil. Moreover, the findings suggest that this culicid species may perform an important role as a vector for *D. immitis* in an area (*i.e.*, Brazil) where greater importance has long been attributed to *A. aegypti*. Field studies should be carried out to clarify the real importance of this culicid species in the transmission of *D. immitis* in Brazil.

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