Vector competence of *Culex quinquefasciatus* Say, 1823 exposed to different densities of microfilariae of *Dirofilaria immitis* (Leidy, 1856)

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ABSTRACT. Vector competence of *Culex quinquefasciatus* Say, 1823 exposed to different densities of microfilariae of *Dirofilaria immitis* (Leidy, 1856). The metropolitan region of Recife, Brazil is endemic for *Dirofilaria immitis* and has an environment favorable to the development of *Culex quinquefasciatus*. The goal of this study was to evaluate the vector competence of the *Cx. quinquefasciatus* RECIFE population for *D. immitis* transmission. A total of 2,104 females of *Cx. quinquefasciatus* RECIFE population were exposed to different densities of *D. immitis* microfilariae blood meals, ranging from 1,820 to 2,900 mf/ml of blood, in a natural membrane apparatus. The results showed a variation between 92.3% and 98.8% of females fed. The exposure of the *Cx. quinquefasciatus* RECIFE population to different densities of microfilariae did not influence the mortality of the mosquitoes. Infective larvae from *D. immitis* were observed in the Malpighian tubules beginning on the 12th day, whereas larvae were observed in the head and proboscis beginning on the 13th day following infection. The vector efficiency index (VEI) presented by the mosquitoes ranged from 7.8 to 56.5. The data demonstrates that the *Cx. quinquefasciatus* RECIFE population has great potential for the transmission of *D. immitis*, as it allowed the development of the filarid until the infectious stage at the different densities of microfilariae to which it was exposed.

KEYWORDS. Biology; experimental infections; mosquitoes.

RESUMO. Competência vetorial de *Culex quinquefasciatus* Say, 1823 exposto a diferentes densidades de microfilárias de *Dirofilaria immitis* (Leidy, 1856). A Região Metropolitana do Recife é endêmica para *Dirofilaria immitis* e possui ambiente favorável para o desenvolvimento de *Culex quinquefasciatus*. Neste estudo avaliou-se a competência vetorial de *Cx. quinquefasciatus* população RECIFE para a transmissão de *D. immitis*. Para tanto, 2.104 fêmeas de *Cx. quinquefasciatus* população RECIFE foram expostas a diferentes densidades de microfilárias de *D. immitis*, variando de 1.820 a 2.900 mf/ml de sangue por meio de membrana natural. Os resultados obtidos demonstraram variação de 92,3% a 98,8% de fêmeas ingurgitadas após a alimentação. A exposição de *Cx. quinquefasciatus* população RECIFE a diferentes densidades de microfilárias não influenciou na mortalidade dos mosquitos. Larvas infectantes de *D. immitis* foram observadas nos túbulos de Malpighi a partir do 12° dia, enquanto na cabeça e na probóscide foram observadas a partir do 13° dia após a infecção. Os índices de eficiência vetorial (IEV) apresentados pelo culicídeo variaram de 7,8 a 56,5. Os dados obtidos demonstraram que *Cx. quinquefasciatus* população RECIFE tem grande potencial para a transmissão de *D. immitis*, pois permitiu o desenvolvimento do filarídeo até o estágio infectante nas diferentes densidades de microfilárias às quais foi exposto.

PALAVRAS-CHAVE. Biologia; infecções experimentais; mosquitos.

Dirofilaria immitis (Leidy, 1856) is a commonly found parasite in the heart cavities and pulmonary arteries of canines and felines, and can also infect humans (Samarawickrema *et al.* 1992). Although the transmission of *D. immitis* is carried out by *Culex* Linnaeus, 1758, *Aedes* Meigen, 1818, *Anopheles* Meigen, 1818, *Mansonia* Blanchard, 1901, *Psorophora* Robineau-Desvoidy, 1827 and *Coquillettidia* Dyar, 1905 species (Ludlam *et al.* 1970), several studies are being conducted in various regions of the world in order to determine the vector species of this nematode.

In Brazil, the species *Cx. quinquefasciatus* Say, 1823 is considered a potential vector for *D. immitis* in the states of Rio de Janeiro, (Lourenço-De-Oliveira & Deane 1995; Labarthe et al. 1998 a,b), Alagoas (Brito *et al.* 1999) and Maranhão (Ahid & Lourenço-De-Oliveira 1999; Ahid *et al.* 2000). In the Brazilian Northeast Region, the species *Cx. quinquefasciatus* is important in locations that are endemic for *Wuchereria bancrofti* (Cobbold, 1877), as it is the main vector of this filarid (Medeiros *et al.* 1992), particularly in the metropolitan region of Recife in the state of Pernambuco, where bancroftian (Albuquerque *et al.* 1999; Lima *et al.* 2003) and canine filariosis, (Alves *et al.* 1999) are endemic.

The goal of the present study was to evaluate the vector competence of the *Cx. quinquefasciatus* RECIFE population

artificially exposed to different densities of *D. immitis* microfilariae.

MATERIAL AND METHODS

Mosquitoes. The mosquitoes used in these experiments were of the species *Cx. quinquefasciatus* RECIFE population which was established in 2003 from eggs provided by Aggeu Magalhães Research Center (FIOCRUZ), Recife, PE. Maintenance of the colony was performed in a climate-controlled room at a temperature of 28 ± 1°C with 80 ± 5% relative humidity and a natural photoperiod cycle at the Insect Laboratory for Parasitic Diseases of Domestic Animals of the Department of Veterinary Medicine of the Universidade Federal Rural de Pernambuco (UFRPE), Recife, PE. Canine blood was offered to the females for feeding once a week in an artificial feeder (Rutledge *et al.* 1964; Mendonça *et al.* 1998).

***D. immitis* microfilariae strain.** The microfilariae used for the experimental purpose were obtained of a domiciliated dog with natural infection from the Metropolitan Region of Recife, Pernambuco, Brazil.

Blood collection. Blood were collected through venous puncture of the cephalic vein, into a test tube containing Ethylenediaminetetraacetic Acid for blood microfilaria count and also in tube containing heparin destined to the infectious feeding of the female mosquitoes.

Processing of the material. The microfilaremia of the animal was quantified using the modified Knott method (Newton & Wrigth 1956).

Experimental mosquitoes exposure to *D. immitis* microfilariae. For the first artificial blood meal, a total of 2,104 *Cx. quinquefasciatus* RECIFE population females between three and seven days of age were used (Rutledge *et al.* 1964). As the membrane for the artificial meal, a quail skin (*Coturnix coturnix* (Linnaeus, 1758)) was choose. The time exposure for each meal was two hours. For this procedure, undiluted dog blood containing five densities of *D. immitis* microfilariae was used. As control group, a total of 1,200 females were fed with non-infected blood (Table I). Prior to exposure, the female mosquitoes were deprived of sugar solution for 24 hours. After the experimental infection, the *Cx. quinquefasciatus* females were fed with a sugar solution and all the non ingurgitated were discarded.

Dissection of mosquitoes and observation of *D. immitis* larvae. For a period of 14 days, ten females per day had the Malpighian tubules, head and thorax extracted in a saline solution, macerated by means of compression between the slide and cover slip, and stained using the Giemsa method to facilitate the microscopic analysis of the *D. immitis* larvae. Observations were made on an optical microscopy and development stages were determined following Taylor (1960).

Analysis of *Cx. quinquefasciatus* vector competence for transmitting *D. immitis*. After two hours of blood meal, the percentage of ingurgitated females was recorded, and ten females of each group were dissected and average number of ingested microfilaria was calculated. Mortality of the culicids was observed daily in a cumulative form and expressed in both absolute and relative values. Dead mosquitoes were dissected and examined for *D. immitis* larvae daily. The infection ratio (IR) was calculated according to procedures described by Ahid *et al.* (2000) and the vector efficiency index (VEI) was determined according to the procedures described by Kartman (1954).

Statistical analysis. The linear regression test was used to assess the influence of the different densities of *D. immitis* microfilaria per milliliter (ml) of blood to which the mosquitoes were exposed as well as the proportion of ingurgitation of the females and differences in ingested microfilaria. The Kruskal-Wallis test was used to assess microfilaria ingestion by the *Cx. quinquefasciatus* RECIFE population at the different densities of *D. immitis* microfilaria (mf) per ml of blood. ANOVA was used to compare larval development until the infectious stage in the culicid females as well as the mortality variation of the mosquitoes at the different densities of mf/ml. The BioStat program (version 2.0) was employed for the statistical calculations (Ayres *et al.* 2000).

RESULTS

Following the feeding of the *Cx. quinquefasciatus* RECIFE population with blood containing different densities of microfilaria/ml, a variation between 92.3 and 98.8% of fed females was observed (Table II). On the control group, almost 100% of ingurgitated was observed. The average number of microfilaria ingested per female ranged from 6.6 to 29.2; the average observed at the 1,820 density differed significantly ($p<0.05$) from averages found for the 1,913 and 2,558 densities.

The mortality rate of the *Cx. quinquefasciatus* RECIFE population did not surpass 36.3%, regardless of the density of *D. immitis* microfilaria in the blood meal (Fig. 1). For the 2,558 microfilaria/ml density, the culicids exhibited a mortality rate and vector efficiency index of 8.9% and 56.5%, respectively. Similar mortality rates (ANOVA $p>0.05$) were found among females exposed to densities of 1,913 and 2,558 mf/ml of blood. These rates were lower than those found for the densities of 1,820, 2,000 and 2,900 mf/ml of blood and did not differ significantly from one another (ANOVA $p>0.05$). The mortality rate among the females of the mosquitoes fed with non-infected blood (control) was lower than that found among the females exposed to *D. immitis* microfilaria (ANOVA $p>0.05$) (Fig. 2).

The development time for *D. immitis* in the *Cx. quinquefasciatus* RECIFE population was similar for all densities of microfilaria. The sausage stage began to be observed on the third day following infection and persisted until the ninth day. The melanization process occurred in 5.4% (106/1,961) larvae of *D. immitis* in the culicids; this reaction

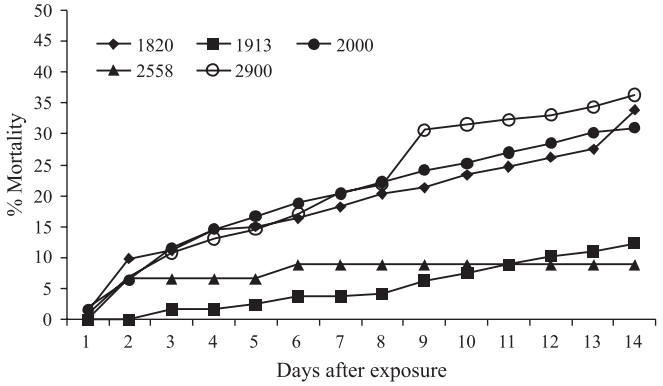

Fig. 1. Accumulative mortality of *Cx. quinquefasciatus* RECIFE population after fed with blood containing five different densities (1,820 to 2,900 mf/ml) of *D. immitis* microfilariae.

Table I. Number of females of the *Cx. quinquefasciatus* RECIFE population fed with blood containing different densities of *D. immitis* microfilaria (experimental group) or non-infected blood (control group).

Density microfilaria/ml	Number of females	
	Exposed	Unexposed (Control)
1,820	302	300
1,913	250	250
2,000	900	300
2,558	52	50
2,900	600	300

wasn't observed by the 14th day for 2,558 mf/ml of blood. At densities of 1,913 and 2,900 mf/ml of blood, one and three first stage larvae (L_1) with altered morphologies were found, respectively. From the sixth to 11th day, second stage larvae (L_2) of *D. immitis* were observed, whereas third stage larvae (L_3) were observed beginning on the 12th day following infection for all densities, except the density of 1,913 mf/ml of blood, which only exhibited infectious larvae on the 13th day.

Culex quinquefasciatus females exhibited infectious larvae (L_3) of *D. immitis* in the Malpighian tubules starting on the 12th day and exhibited infectious larvae in the head and proboscis on the 13th day following infection. The vector efficiency index (VEI) for the *Cx. quinquefasciatus* RECIFE population was from 7.8 to 56.5% (Table II). Regarding the infection ratio, the highest index was found in the group of females exposed to a density of 1,913 microfilaria/ml (Table III).

DISCUSSION

In regarding of the *Cx. quinquefasciatus* infection by *D. immitis*, it is known that *Cx. quinquefasciatus* can ingest large quantities of microfilaria. Due to physiological and immunological reactions, however, they may limit the number of *D. immitis* that will develop to the infective stage. This strategy ensures the survival of the culicid, as the development of a large number of nematode larvae destroys the Malpighian tubules of the mosquito, thereby causing high mortality (Palmer *et al.* 1986). The results regarding the average of microfilaria ingested (28.3) by the *Cx. quinquefasciatus* RECIFE population at a density of 2,558 mf/ml were higher than those observed by Lai *et al.* (2000) for the Taiwan population (15.3).

The *Cx. quinquefasciatus* RECIFE population demonstrated vector efficiency, as the mortality rate of the culicid was not influenced by the exposure of different densities of *D. immitis* microfilaria ($F = 0.0615$, $p>0.05$). According to Scoles (1998), a culicid must resist infection regardless of the parasitic load; survive long enough to permit the development of the larvae until the infectious form for mammals; feed on canine blood; be adapted to the geographic region; be abundant; and exhibit various population peaks throughout the year (Ludlam *et al.* 1970; Christensen 1977). Thus, the *Cx. quinquefasciatus* RECIFE population exhibits the necessary characteristics to be considered competent in the transmission of *D. immitis.*

It is also known that infected mosquitoes are more sensitive to temperature variations, with diminishing survival rates at higher temperatures (Kutz & Dobson 1974). However, the main cause of mortality of infected females is attributed to the development of *D. immitis* in the mosquito, with peak mortality generally occurring within the first 48 hours after infection, when first stage larvae (L_1) of the nematode invade the Malpighian tubules of the females and later, between the 12th and 14th day, break free from these tubules and migrate to the head and proboscis of the culicids (Kartman 1953; Buxton & Mullen 1981).

The development time for *D. immitis* in the *Cx. quinquefasciatus* RECIFE population was similar at all microfilaria densities, which is in agreement with data obtained by Ahid *et al.* (2000) for *Cx. quinquefasciatus* and is also similar to results obtained by Taylor (1960) and Mendonça *et al.* (1998) for *Ae. aegypti*. However, it was observed that melanization occurred in some *D. immitis* larvae, thereby emphasizing the restrictions that the culicid population placed

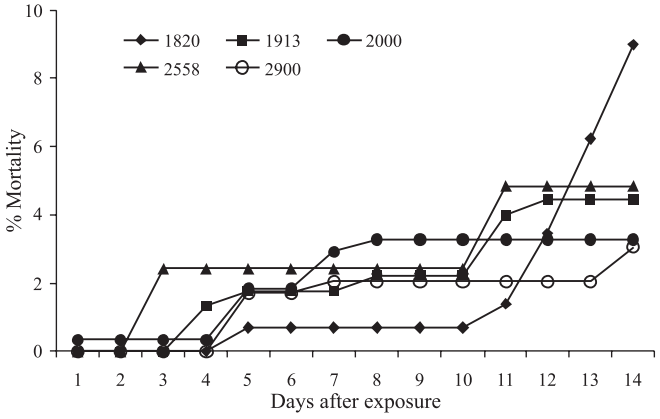

Fig. 2. Accumulative mortality for the five control groups of *Cx. quinquefasciatus* RECIFE population fed with healthy dog blood (related to experimental groups fed with 1,820 to 2,900 mf/ml).

Table II. Percentage of females of the *Cx. quinquefasciatus* RECIFE population ingurgitated with different densities of *D. immitis* microfilaria/ml of blood, with respective average of microfilaria ingested (mf), third stage (L_3), and Vector Efficiency Index (VEI).

Density microfilaria /ml	Percent females ingurgitated	$\bar{x}$ mf ingested*	$\bar{x}$ L_3**	VEI (%)
1,820	94.7	6.6 ± 8.0 [a]	2.2 ± 1.4 [A]	33.3
1,913	93.6	29.2 ± 11.7 [b]	2.3 ± 1.9 [A]	7.8
2,000	94.8	17.7 ± 14.5 [a]	1.8 ± 1.6 [A]	10.2
2,558	92.3	28.3 ± 4.1 [b]	16.0 ± 2.6 [B]	56.5
2,900	98.8	16.5 ± 8.5 [a]	2.2 ± 1.4 [A]	13.3

* average number of microfilaria ingested – observed immediately following blood meal.
** average number of L_3 *D. immitis* in female mosquitoes. Values represented by letters in the columns are statistically different.
mf – microfilaria.

Table III. Infection ratio (%) of *Cx. quinquefasciatus* RECIFE population experimentally exposed to different densities of *D. immitis* microfilariae.

Density microfilaria/ml	Number of females		Infection Ratio (%)
	Dissected	Infected	
1,820	276	85	30.8
1,913	224	105	46.8
2,000	824	111	13.5
2,558	45	18	40.0
2,900	573	146	25.5

on the nematode regarding its development. According to McGreevy *et al.* (1978) and Coluzzi *et al.* (1982), *Cx. quinquefasciatus* eliminates a large number of ingested microfilaria by means of imprisonment in the cibarium and later destruction as well as by melanization mechanisms (Christensen & Forton 1986), the rapid coagulation of ingested blood, and the presence of oxyhemoglobin crystals in the intestinal content resulting from the oxidation of the ingested blood (Nayar & Sauerman 1975; Lowrie 1991; Loftin *et al.* 1995). This allows the development of a supportable number of larvae that do not cause much damage.

The females of *Cx. quinquefasciatus* exhibited infectious larvae in the Malpighian tubules on the 12th day and in the head and proboscis on the 13th day pos infection. Brito *et al.* (1999) observed infectious larvae in the Malpighian tubules of the *Cx. quinquefasciatus* ALAGOAS population on the 14th day, whereas Ahid *et al.* (2000) found infectious larvae (L_3) in the Malpighian tubules of the *Cx. quinquefasciatus* RECIFE population on the 11th day and in the proboscis only on the 14th day following infection. The detection periods of the larval stage found in the present study are in agreement with reports by Taylor (1960) and Mendonça *et al.* (1998) for *Ae. aegypti* Linnaeus, 1762.

The fact that the infection ratio in the *Cx. quinquefasciatus* RECIFE population was not influenced by the feeding rate of the females (F=3.2937, p>0.05) and did not increase with the increased number of *D. immitis* microfilaria ingested (F = 2.1952, p>0.05) is similar to observations by Russell & Geary (1996), who found no relationship between the increase in the density of *D. immitis* microfilaria and the infection ratio of *Cx. annulirostris* Skuse, 1889. In the present study, the number of *D. immitis* larvae having developed to the infectious stage in *Cx. quinquefasciatus* was also not influenced by the different densities of microfilaria (F=0.5912, p>0.05) to which the mosquitoes were exposed, nor by the increase in the number of microfilaria ingested (F=1.3569, p>0.05).

The data obtained demonstrate that the *Cx. quinquefasciatus* RECIFE population exhibits great potential for the transmission of *D. immitis*. It withstood the development of the nematode through to the infectious stage at the different densities of microfilaria to which it was exposed. Labarthe *et al.* (1998a) considers *Cx. quinquefasciatus* a secondary vector in both Rio de Janeiro as well as São Luis, MA. Ahid *et al.* (1999) found this species naturally infected by parasite larvae in the infectious stage, suggesting that it is a good *D. immitis* vector in Northeast Brazil. The results from the present study broaden this possibility. By means of experimental infections, the *Cx. quinquefasciatus* RECIFE population proved to have competence for the transmission of *D. immitis,* suggesting that it may be one of the transmission species of this nematode in the metropolitan region of Recife, Brazil.

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