Journal of the Egyptian Society of Parasitology, Vol.43, No.3, December 2013
J. Egypt. Soc. Parasitol., 43(3), 2013: 591 - 600

HISTOLOGICAL AND PCR XENOMONITORING OF CULICINE MOSQUITOES FOR FILARIAL INFESTATION IN SOHAG GOVERNORATE, UPPER EGYPT

By

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Abstract

The nematodes *Wuchereria bancrofti* and *Brugia malayi*; the principal etiologic agents of lymphatic filariasis are mosquito dependant in the biological transmission. Dirofilariasis is essentially a disease of canines which can also be transmitted to humans by culicine mosquitoes.

The present work studied the histological and PCR xenomonitoring filarial infestation in culicine mosquitoes in Sohag Governorate. One hundred female mosquitoes of the 5 culicine spesies present in the selected localities, were dissected and histological sections of thoracic muscles were examined for filarial larvae. Also 50 female *Culex pipiens molestus* were collected from the same areas and tested for 3 filarial worms using PCR. *Cx. pipiens molestus* was the only culcine mosquito harbouring larvae of *Wuchereria bancrofti* and *Dirofilaria* spp. Results were compared and it was found that PCR proved easier to do and it gave better data as it was able to differentiate dirofilarial species. The results indicated a clear success of health authorities in anti-filariasis control measures and pointed out to avoid the hazard of possible occurrence of future cases of human dirofilariasis in Sohag Governorate.

Key words: *Wuchereria bancrofti,Dirofilaria* spp, *Culex pipiens molestus,* PCR.

Introduction

Mosquitoes are unquestionably the most medically important arthropod vectors of disease. The maintenance and transmission of the pathogens that cause malaria, lymphatic filariasis, and numerous viral infections are absolutely dependent on the availability of competent mosquito vectors. The nematodes *Wuchereria bancrofti* and *Brugia malayi* are the principal etiologic agents of lymphatic filariasis. (Bockarie *et al*, 1998). The disease can lead to chronic, disabling conditions such as lymphedema, elephantiasis and genital deformities. It is one of the major vector-borne diseases affecting over 100 million people in 73 countries in tropical and subtropical areas worldwide (Helmy *et al*, 2004).

In the Nile Delta of Egypt periodic bancroftian filariasis is focally endemic, especially in the governorates of

Qalyubiya, Sharkiya, Dakahliya and Damietta where the main mosquito vector, *Culex pipiens*, is abundant. In contrast, filarial infections was not reported from most of middle and upper Egypt, with the exception of small hypoendemic areas in Assiut and Sohag governorates (Harb *et al*, 1993).

The only study detecting the role played by mosquitoes in transmission of filariasis in Sohag Governorate was reported in unpublished M.Sc. Thesis in as early as 1987 by Ebraheem who stated that El- Atamna village in Tema district, Aksas village in El-Maraga district, Akhmeem city in Akhmeem district and El- Zara village in El-Monshah district were positive areas for filariasis

The present study aimed at xeno-monitoring culicine mosquitoes in Sohag Governorate for filarial infestation by both thoraces Hematoxylin and Eosin stained histological sections and PCR.

Material and Methods

The present study was done in Sohag Governorate, one of the Governorates of Upper Egypt located about 470 kilometer to the south of Cairo. The study was carried out in 4 districts; Tema, El Maraga, Akhmeem and El Monshah.

Examination of mosquitos thoracic muscles for filarial infection: One hundred female mosquitoes from the 5 culicine species previously detected in the same examined locality by Khalifa *et al*. (2013); namely: *Culex pipiens molestus* (Forskal 1775)*, Cx. antennatus* (Beker 1903)*, Cx. pusillus* (Macquart 1850), *Aedes caspius* (Pallas 1771) and *Culiseta longiareolata* (Macquart, 1902) were collected from July 2009 to September 2010. Thoraces were prepared for histological examination after Bancroft and Gamble (2002).

PCR based technique: A total of 50 female *Cx. pipiens molestus* (the only species found infected with filarial larvae by dissection) were collected from each of the four research districts, pooled separately and prepared for conventional and multiplex PCR as recommended by the manufacturer (Qiagen, Hilden, Germany). The PCR procedure for detection of *W. bancrofti* in mosquito vectors was executed according to Siridewa *et al.* (1996) using forward pWb12 F (5`-CTGAGTGAA ATCAATGAACTGC) and reverse pWb12 R (5`-GTCCATCCGATGAAG TTCCACC) primers.

The PCR procedure for detection *Dirofilaria immitis* in mosquito vectors was done as described by Rishniw *et al*. (2006). The primers used were forward DI COI-F1 (5`-AGTGTAGAGG GTCAGCCTGAGTTA-3`) and reverse DI COI-R1 (5`-ACAGGCA-CTGACA ATACCAAT-3`)

The PCR procedure for detection of *Dirofilaria repens* in mosquito vectors was done as described by Latrofa *et al.,* (2012). The primers used were Forward Dr ITS2-F (5`-CATTGATAG TT TACATTCAAATAA-3`) and reverse Dr ITS2-R (5`-GATTCATTTA TTG CATTAAGCAAGC-3`) and the PCR products of the 3 procedures were detected by standard techniques of agarose gel electrophoresis. DNA was visualized by an ultraviolet transillumina-

tor following staining the gel with ethidium bromide.

Results

Cut sections of filarial larvae were found parallel to thoracic myofibrils with noticeable separation between the filarial larvae cuticle and the mosquito myofibrils suggesting physical breaks caused by the migrating larvae toward the head by laterally crossing the myofibers before exiting into the periphery of the thorax (Fig.1,a). They were 1000-1600µ in length with a cuticle showing the obvious muscle layer (Fig.1,b).

Microscopic examination of caudal ends of the detected larvae showed the presence of two different types (Fig. 2, a,b). The caudal end of one of them had three rounded buble-like terminal protuberances (fig.3,a) suggesting that these are the larvae of *Wucheraria bancrofti* (WHO, 1983) . In the other type, the caudal end was cigar-shaped with minute terminal papillae (Fig. 3,b) suggesting that these larvae were belonging to genus *Dirofilaria* (WHO, 1983). Cocerning *W. bancrofti,* four mosquitoes were found infected (all from El-Maraga district) while *Dirofilaria* larvae were found in three mosquitoes; one from Akhmeem and two from El-Maraga districts (Tab. I).

PCR was done for detection of *W. bancrofti* , *Dirofilaria immitis* and *D. repens* in *Cx. pipiens molestus* collected from the same four disticts. Positive results are shown in (Tab. II).

For *W. bancrofti*, amplification of a band of expected size was exclusively observed in the two pools of El Maraga district and Akhmeem district (Fig. 4, a). For *Dirofilaria immitis*, amplification of a band of the expected size was exclusively observed in the pool of El Maraga district only (Fig. 4, b). Multiplex PCR results (Fig. 5) confirmed the above mentioned results.

Table I: Rate of filarial infection in histologically examined mosquitoes.

Mosquito type	Mosquito dissected	Mosquitoes with *W. bancrofti* larvae	Mosquitoes with *Dirofilaria* larvae	Total
Cx. pipiens molestus	72	4	3	7
Cx. antennatus	3	___	___	___
Cx. pusillus	7	___	___	___
Ae. caspius	8	___	___	___
Cs. longeareolata	10	___	___	___
Total	100	4	3	7

Table II: PCR results

parasite / District	*W. bancrofti*	*D. immitis*	*D. repens*
Tema district	Positive	--------	--------
El Maraga district	Positive	--------	--------
Akhmeem district	---------	Positive	--------
El Monshah district	----------	---------	--------

Discussion

From the five studied culicine mosquitoes, only *Culex pipiens molestus* was found to harbour filarial larval infestation. Four out of 100 examined *Cx. pipiens* female mosquitoes were found infected with *W. bancrofti* during examination of mosquitoes' thoraces; all were in El Maraga district.

El-Nadi *et al.* (2004) exihibited the unpublished results of the M.Sc. Thesis of Ibraheem which was done in 1987 about the role played by mosquitoes in transmission of bancroftian filariasis in Sohag Governorate. His study was limited to detection of *W. bancrofti* in mosquitoes and by dissection only. He found 30 (0.44%) out of 6757 of dissected *Cx. pipiens molestus* infected with the filarial larvae with 0.27% incidence (5 infected out of 1846) in Tema, 0.48% incidence (8 infected out of 1665) in El Maraga, the incidence was 0.64% (10 infected out of 1547) in Akhmeem and 0.41% (7 infected out of 1699) in El Monshah. It is worth mentioning that the present authors are in doubt that some of his larvae may be those of *Dirofilaria* spp.The fact that he reported bancroftian filariasis in the four districts which is not in agreement with the present results where this parasite was revealed only in El-Maraga district may be explained by the presence of a high rate of actively infected patients with abundant nocturnal microfilariae in their peripheral blood at the time of his study or by sucessful control efforts performed by the Menistry of health during the past 26 years since his study was done.

Cx pipiens was found to be the most efficient natural vector of *W. bancrofti* in Egypt (Farid *et al*, 2000) and the most reliable microscopic distinguishing feature is the caudal end of the parasite; *W. bancrofti* larvae have three distinctive bubble like protuberances (WHO, 1983).

Comparing the microscopic and PCR results for *W. bancrofti*, we found that PCR has detected positive results in both El Maraga and Tema districts adding a new district (Tema) to that detected by microscopic examination of mosquitoes thoraces making PCR more accurate in detection of the mosquito infection.

PCR was reported by many authors to be of great benefit in detection of bancroftian filariasis infestation in its mosquito vector and infection in humans. Thus, Farid *et al.* (2001) recommended the use of PCR for evaluation of *W. bancrofti* in different Egyptian villages. Williams *et al.* (2002) used PCR in mosquito pools as a promising tool for monitoring the progress to eliminate the disease. Rao *et al.* (2006) believed that the real-time PCR has great potential as a tool for monitoring bancoftian filariasis in mosquito tissues and human blood. Boakye *et al.* (2007) introduced some improvement of PCR-based pool screening for *W. bancrofti* so as to detect as low as one 3rd stage larva in pools of 25-100 mosquitoes. Mishra *et al.* (2007) developed and evaluated a single multiplex PCR for simultaneous detection of *Brugia malayi* and *W. bancrofti.*

The control efforts done allover last years to eliminate the bancroftian filariasis from Egypt by the wide spread use of diethylcarbamazine citrate treatment and the intensive use of residual insecticides for mosquito control, has made bancroftian filariasis endemic in limited foci in Egypt, mostly in the Nile river Delta region (Lower Egypt) and in Giza (Ramzy *et al,* 2006).

Out of 100 mosquitoes, three *Cx. pipiens molestus* female mosquitoes were found infected with *Dirofilaria* during examination of mosquitoes' thoraces one in Akmeem district and two in El Maraga district. This finding does not represent a a small percentage to be neglected for a zoonotic disease especially when the present authors were examining mosquitoes and not clinically ihfected humans or dogs.

Cx pipiens was found to be the most efficient natural vector of *D. immitis* and *repens* (Capelli *et al*, 2013) and the most reliable microscopic distinguishing feature is the caudal end of the parasite; *Dirofialia* larvae have cigar shaped tail with minute terminal papillae. However, by this method it was not possible to identify *Dirofilaria* larvae at the species level (WHO, 1983).

Comparing the microscopic and PCR results for *Dirofilaria*, the present authors found that PCR has detected positive presence of *D. immitis* in mosquitoes from Akhmeem district only, while the histological examination revealed the presence of infested mosquitoes in both Akmeem and El-Maraga districts.

For the identification of dirofilarial infections in its mosquito vector, there was much controversy between using stained thoracic sections and the use of the different procedures of PCR particularly in cases of multiple microfilarial infestation (Mar *et al*, 2002). Cancrini *et al.* (2006) found that DNA evidenced the presence of *D. immitis* in rural areas whereas in urban areas infective larvae of *D. repens* were detected. Rivasi *et al.* (2006) were able to detect *D. repens* by PCR in embeded parafin tissues from two human pulmonary locations. Hence, it is advised to use their technique when it is impossible to identify the worm species by conventional morphology. Thanchomnang *et al.* (2010) used the real-time flourescence resonance transfer PCR and melting curve analysis for rapid identification of *D. immitis* in mosquito vectors and dogs. They concluded that their procedure was a suitable and powerful tool for epidemiological surveys of canine dirofilariasis as well as for molecular xenomonitoring of *D. immitis* in mosquitoes. Licitra *et al.* (2010) used PCR for detection of *D. immitis* in mosquitoes in Georgia (USA) by using primers of the worm surface or cuticular antigens and reported *Aedes albopictrus* as a siutable mosquito for the parasite development. Vezzani *et al.* (2011) screened the occurrence of *D. immitis* in South America and could identify the parasite in *Cx. pipiens* and *Aedes egypti* while Latrofa *et al.* (2012) used multiplex PCR for simultaneous

detection of filarial species infecting dogs in Italy.

The present results indicated that PCR above being easier, time and effort saving than microscopic examination, the technique also detected the *Dirofilaria* species as *D. immitis* which was impossible by routine microscopic examination. However, PCR was negative both for *D. immitis* and *D. repens* in El Maragha district while the microscopic examination showed one *Dirofilaria* infected female mosquito. This can be explained by the presence of 30 different species of *Dirofilaria*; *D. repens* and *D. immitis* are the 2 most common species that frequently infect humans (De *et al*, 2012) the only medically important two searched for species during this work.

Many cases of human dirofilariasis were reported from Egypt (Awadalla *et al*, 1998; Munichor *et al*, 2001; Antonios and Bayoumy, 2002, Maher *et al*, 2004, Abdel-Rahman *et al,* 2008; El-Nadi and Abdel-Nour, 2009).

Elsayed *et al.* (2012) mentioned that the increase in the number of cases of dirofilariasis, which were previously considered exceptional, resulted from more careful examination and more refined diagnostic techniques; yet the increase did seem to be real. To the best of the authors knowledge, the present study was the first screening for *Dirofilaria* infected mosquitoes in Upper Egypt. Detection of human infections with dirofilariasis in Egypt and in two Governorates of Upper Egypt makes it worth to study dirofilariasis in stray dogs of Sohag Governorate.

Conclusion

The present study compared histological thoracic sections and PCR as a tool for xenomonitoring filarial infection in mosquitoes in four districts in Sohag Governorate and explained the advantages and limitations of both techniques. The present study also updates the knowledge about the role of culicine mosquitoes in transmission of filarial diseases in the governorate. Of course, the present results indicated a clear success of health authorities in Sohag in anti-filariasis control measures, but pointed out to avoid the hazards of the possible increase of future cases of human dirofilariasis.

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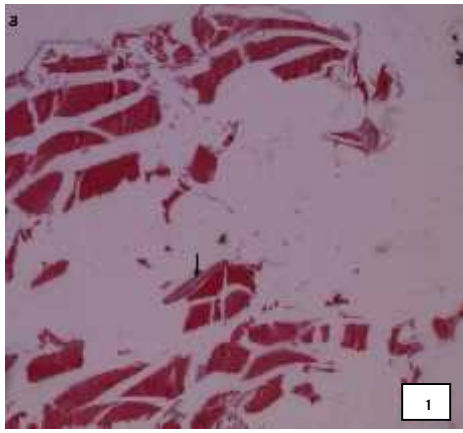
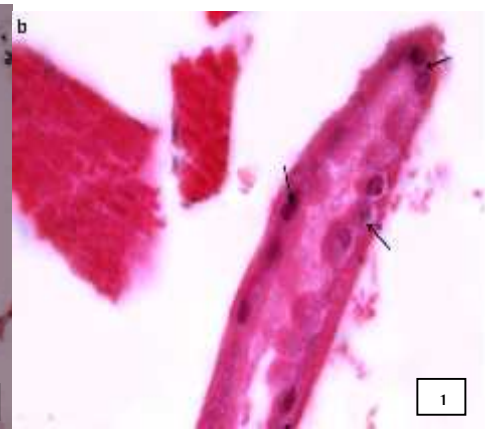
Explanation of figures

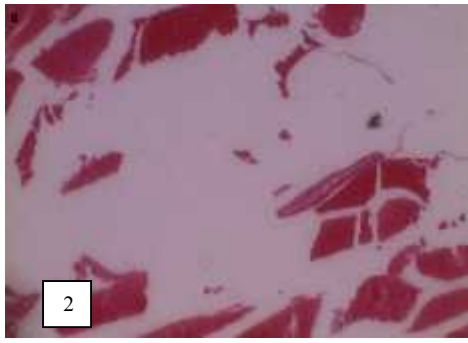
Fig. 1a: Larva in mosquito tharacic muscles stained with H & E. Larvae parallel to myofibrils with noticeable separation between filarial larvae cuticle and mosquito myofibrils. (b) Muscle layer of larva.

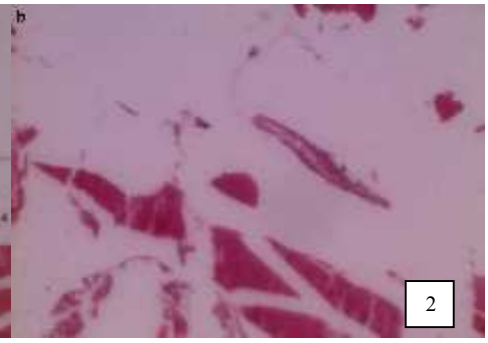
Fig. 2: Two types of larvae identified (a) *W. bancrofti* larva (b) *Dirofilaria* larva.

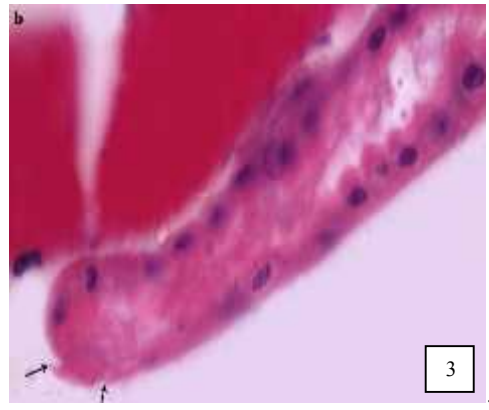
Fig. 3a: Caudal end of *W. ancrofti* larvae with three buble like terminal protuberance. (b) Caudal end of *Dirofilaria* larvae showing terminal papillae











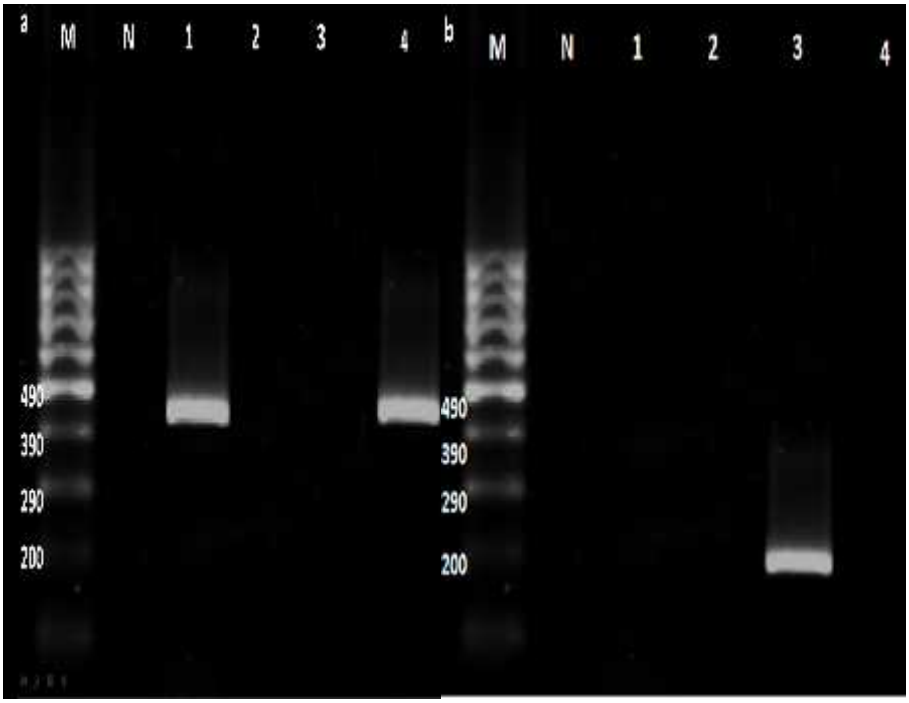


Fig. 4: N: Negative control, Lane1 DNA of mosquitoes collected from El Maraga , Lane 2 from El Monshah, Lane 3 from Akhmeem and lane 4 from Tema. (a) positive PCR for *W. bancrofti* in El Maraga distict and Tema. (b) positive PCR for *Dirofilaria immitis* in Akhmeem district.

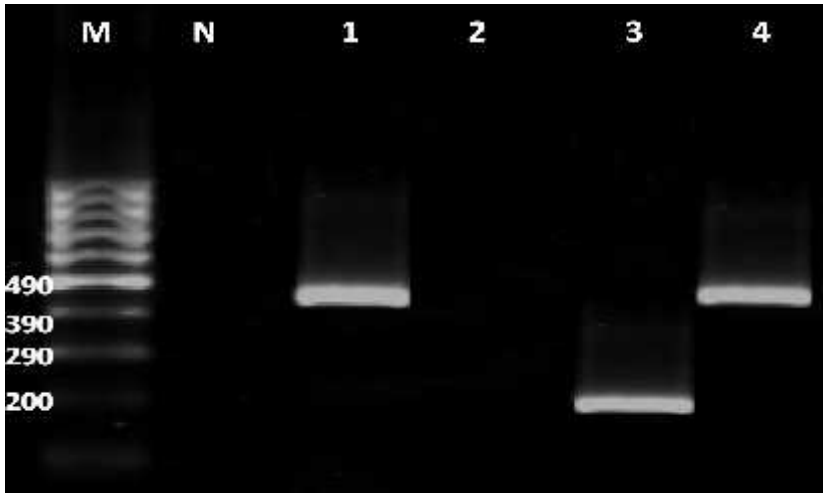


Fig 5: Multiplex PCR. N: Negative control, Lane1 positive results for *W. bancrofti* in El Maraga distict , Lane 2 from El Monshah, Lane 3 positive results for *Dirofilaria immitis* in Akhmeem district and lane 4 from Tema positive results for *W. bancrofti*