MOSQUITO IDENTIFICATION AND MOLECULAR XENOMONITORING OF LYMPHATIC FILARIASIS IN SELECTED ENDEMIC AREAS IN GIZA AND QUALIOUBIYA GOVERNORATES, EGYPT

By

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Abstract

Lymphatic filariasis is a vector-borne health problem that has been focally endemic in Egypt for centuries. The chief vectors of transmission are Culicinae species. Control measures in the form of mass drug administration of DEC citrate treatment have been implemented in Nile delta for almost a decade. This study aimed to identify the prevalent mosquito species in endemic areas in Giza and Qualioubiya governorates and to monitor *Wuchereria bancrofti* infection by detecting the parasite DNA in collected mosquitoes. Adult mosquitoes were collected using light traps hung indoors. Microscopic examination was performed to identify and examine the morphologic characters of mosquitoes. Female *Culex* mosquitoes were subjected to semi-nested PCR to detect filarial DNA targeting repetitive DNA sequences (pWb12 repetitive region) specific for *W.bancrofti*.

  The results revealed 3 species of mosquitoes *Culex pipiens, Culex pusillus and Culex quinquefasciatus* with the predominance of *Culex pipiens* (85.7%). *Wuchereria bancrofti* DNA was not detected in any of the collected mosquito pools. With the progress of elimination programme in Nile Delta, follow up studies with larger sample size are recommended as the predominance of *Culex pipiens* the main lymphatic filariasis vector remains a risk of transmission in such areas.

Key words: Egypt, *Culex* species, *Wuchereria bancrofti*, Semi-nested PCR.

Introduction

Lymphatic filariasis (LF) is a debilitating disease with a wide range of clinical manifestations in humans. It is among the neglected tropical diseases and is more common in disadvantaged populations lacking sanitation services (Hotez *et al,* 2012; WHO, 2013a). LF is caused by filarial parasites, mainly *Wuchereria bancrofti* (*W. bancrofti*) with nearly 1.2 billion people living in endemic areas, where they are at risk of infection, and 120 million having the clinical disease worldwide (Foo *et al,* 2011). The infection is endemic in tropical and subtropical areas of Asia, Africa, the western Pacific, and some parts of the Americas and shows a highly focal distribution within an endemic area (Bockarie and Molyneux, 2009).

Several vector types are involved in filariasis transmission, including mosquitoes of the genera *Culex*, *Anopheles*, *Aedes*, *Och-lerotatus* and *Mansonia* with over 100 species (Bockarie and Molyneux, 2009). Globally, the majority of *W. bancrofti* infections are transmitted by *Culex quinquefasciatus* (*Cx. quinquefasciatus*); the chief vector in India, Asia and the Americas. *Anopheles* spp. and *Aedes* spp. mosquitoes are the main vector in Sub-Saharan Africa and the western Pacific respectively, while *Culex pipiens* (*Cx. pipiens*) is the main vector in Egypt and the Middle East (Farid *et al,* 2000; Zagaria and Savioli, 2002; Kaliwal *et al,* 2010; Service, 2012).

Since 1997, the World Health Assembly set the year 2020 as the target for LF elimination as a global public health problem, the WHO has been engaged to meet this objective (WHO, 2010). Egypt was among the first countries to join the WHO global efforts. The programs for the elimination of bancroftian filariasis that have been imple-

mented in the Nile delta sought specifically to decrease microfilaria prevalence rates to less than 0.1% through MDA of an annual dose of DEC (6 mg/kg) in combination with albendazole (400 mg). Administration of these once-yearly, single-dose regimens to people in at-risk communities for 4–6 years makes feasible the prospect of interrupting transmission and thereby eliminating LF (Ramzy *et al,* 2005; El-Setouhy *et al,* 2007; Goldman *et al,* 2007)

Detection of filaria parasites in mosquitoes, often called xenomonitoring, is an epidemiological tool that is increasingly used to detect recurrence of new infections during post-MDA surveillance (WHO, 2013b). Detection of filaria parasites in mosquitoes, often called xenomonitoring, is an epidemiological tool that is increasingly used to detect recurrence of new infections during post-MDA surveillance (WHO, 2013b). PCR proved to be a powerful tool for detection of filarial DNA in infected mosquitoes which provides a practical alternative to conventional dissection methods for the diagnosis of *W. bancrofti* in mosquitoes (Goodman *et al,* 2003; Schmaedick *et al,* 2014).

This study aimed to identify and examine the morphologic characters of the mosquito species prevalent in endemic areas in Giza and Qualioubiya governorates and to monitor *W. bancrofti* infection by detecting the parasite DNA in collected mosquitoes.

Materials and Methods

The laboratory work in this study was carried out in the Lab of Molecular Medical Parasitology (LMMP), Medical Parasitology Department, Faculty of Medicine, and Entomology Department, Faculty of Science, Cairo University and in the Malaria and Emerging Parasitic Diseases Laboratory, National Microbiology Center, Instituto De Salud Carlos III in Spain.

Study sites: Adult mosquitoes were collected from randomly selected houses located in endemic areas, from Bani Salama Village in Giza in July 2014 and from Kafr Ammar and Barshoum Villages in Qualoubia governorates during December 2014 to January 2015.

Mosquito sampling: Adult mosquito collection was done using light traps which were hung indoor, left overnight and collected in the morning. Mosquitoes were knocked down by placing the nets with the trapped mosquitoes at -20^0C for 15 minutes. Afterwards, mosquitoes were transferred into clean zip plastic bags that were closed tightly and labeled with date and place of collection and brought to the laboratory for further processing.

Microscopic examination and mosquito identification: Each mosquito pool was emptied in a Petri dish and examined with the stereomicroscope OLYPMUS$^®$ (SZ9). Female *Culex* mosquitoes were counted, arranged into 70 pools each containing 15-20 mosquitoes that were labeled and kept in freezer at -20◦C for the molecular assay. Representative sample of female *Culex* mosquitoes from collected pools, male *Culex* mosquitoes and other dipterous insects were preserved in 70% ethanol. Female *Culex* mosquitoes were identified until genus level according to Harbach (1985) and Service (2012). Specific identification was carried out according to the combination of Kirkpatrick (1925) and Harbach (1985), and with the aid of Adham (2009) and Ebrahim and Salem (2010). Furthermore, collected mosquitoes were compared to preserved mosquito specimens from two Egyptian insect depositories, which are: the collection of Cairo University, Faculty of Science, Department of Entomology (CUC) and the collection of Research Institution of Medical Insects. The figures of diagnostic characters

were extracted, with some modifications, from Harbach (1985).

Molecular assay for the detection of *W. bancrofti* DNA: Genomic DNA extraction: Genomic DNA extraction was performed on female *Culex* mosquitoes using DNeasy Blood and Tissue Kit, Qiagen, Germany. Mosquitoes were homogenized with the aid of a sonicator in a 1.5ml tube containing 180μl phosphate buffered saline (PBS) and genomic DNA extraction was done according to manufacturer instructions.

Semi-nested PCR and detection system: Extracted DNA from female *Culex* pools were subjected to semi-nested PCR targeting repetitive DNA sequences (pWb12 repetitive region) specific for *W. bancrofti* according to Kanjanavas *et al.* (2005) using three primers: forward primer; WbF (5'-CACCG-GTATCGAGATTAATT-3') and WbR (5'-TTGTTCCTCTATTTGAGACC-3') and Wb2 (5'-TGGATGTATGTCAAAAAGCA-3') as reverse primers. Semi-nested PCR was first performed with an inhibition control in each PCR reaction tube as several studies showed that DNA extracts from mosquitoes may still have some PCR inhibitors that are not completely removed leading to false negative results (Goodman *et al*, 2003; Plichart *et al*, 2006; Beckmann and Fallon, 2012).

The first and second PCR reactions conditions were optimized in 25μl volume. The first reaction contained 12.5μl of Dream Taq Green PCR Master Mix (2x), 0.1μl Dream Taq Polymerase enzyme (5U/μl), 1μl of WbF, 1μl of WbR, 5μl of genomic DNA, 5μl of 10^{-5} inhibition control and 0.4μl of sterile dd H_2O. The PCR amplification was performed using the Biometra TPersonal thermocycler, consisting of initial denaturation at 95°C for 4 minutes, 30 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute and extension at 72°C for 1.5 minutes, finally extension at 72°C for 10

minutes. The expected fragment length is 780 bp. For the second reaction, Dream Taq Green PCR Master Mix, Dream Taq Polymerase enzyme and WbF were the same as the first reaction with addition of 1μl of Wb2, 1 μl of product from the first PCR 9.4 μl of sterile dd H_2O. The second PCR conditions were as follows: initial denaturation at 95°C for 4 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds followed by final extension at 72°C for 10 minutes. The expected fragment length was 400bp.

PCR products were loaded on a 1.5% agarose gel and visualized using UV light. DNA samples that have PCR inhibitors were detected and treated by repeating the DNA extraction for the DNA samples after completing their volume to 200μl by adding PBS. Another semi-nested PCR with the inhibition control was performed on the treated samples to ensure removal of inhibitors. Finally, the mosquito DNA samples were subjected to semi-nested PCR without the inhibition control which was replaced by sterile ddH_2O, with the rest of components and conditions of both the first and second reactions exactly as described before.

Results

Microscopic examination of collected mosquitoes indicated the presence of 3 Culicinae species; *Cx. pipiens* (85.7%), *Cx. pusillus* (7.2 %) & *Cx. quinquefasciatus* (7.1%). The diagnostic characteristics of the genus *Culex* in the examined mosquitoes are a combination of the following: trilobed scutellum (Fig. 1A), absence of both spiracular and postspiracular setae, the presence of one or more lower mesepimeral setae (Fig. 1B), cell R_2 of the fore wing at least as long as vein $R_{2,3}$ (Fig. 1E), tarsomere 1 of fore- and midlegs no longer than tarsomeres 2-5 combined (Fig.1C), hindungues small and inconspicuous (Fig. 1D) and presence of pulvilli

(Fig. 1F). *Cx. pusillus* was identified by a proboscis which is shorter than forefemur, which is a diagnostic characteristic for subgenus Barraudius to which this species belongs (Fig. 2A). on the other hand, *Cx. pipiens* and *Cx. quinquefasciatus* share many characteristics, which are: the absence of both prealar scales and postspiracular scale (Fig. 2B), tarsi usually dark (especially the hind ones), fore-femora, mid-femor and all tibiae clothed with dark scales (Fig. 2C & D), unbanded abdominal sterna with dark scales confined to midline and/or posterolateral corners when present (Fig. 2E), basal pale bands on abdominal terga (Fig. 2F), forecoxa with some dark scales (Fig. 2G), and entirely dark-scaled wing (Fig. 2H). These two species were differentiated by the subcosta which intersects the costa at or beyond level of furcation of $R_{2,3}$ in *Cx. pipiens* (Fig. 2I), while in *Cx. quinquefasciatus* the subcosta intersects costa at or before level of furcation of $R_{2,3}$ (Fig 2J). Other dipterous flies found in mosquito net included *Culicoides circumscriptus, Simiulium griseicolle, Musca domestica, Musca vetripennis, Psychodidae (sand fly),* in addition to some microlepidopteran families.

Out of 70 pools of collected female *Culex* mosquitoes, inhibition was detected in 20 pools. These 20 DNA samples were subjected to treatment to remove the inhibitors; 13 samples were effectively treated while 7 samples remained with inhibition and were excluded from the study. A total of 63 pools were subjected to semi-nested PCR targeting *W. bancrofti* repetitive sequences. *W. bancrofti* DNA was not detected in any of the pools, all were negative.

Discussion

In the present study, the identification strategy of *Culex* species has relied on the overall similarity (i.e. weighting of characteristics among different species) based on very fine morphologic characteristics in proboscis, wings and legs, which often raise the problematic taxonomic issue of presence of intermediate characteristics due to hybridization, for example the very high rate of hybridization between the *Cx. pipiens* and *Cx. quinquefasciatus* (especially in the Arabian Peninsula) make the differentiation between both species using wing characteristics very difficult (Harbach, 1925). Due to this complexity, *Cx. quinquefasciatus* is usually treated under *Cx. pipiens* complex especially in non-taxonomic studies (Smith and Fonseca, 2004; Becker *et al*, 2012). Three species of mosquitoes were identified in the collected sample; *Cx. pipiens, Cx. pusillus and Cx. quinquefasciatus* with the predominance of *Cx. pipiens*. Due to the fact that the mosquito samples were collected from narrow geographical regions, and within a short time period it was not expected to collect a wide variety of mosquitoes. Previous studies reported that *Cx. pipiens* is the main LF vector and the most predominant mosquito species in Egypt. Other Culicinae species were reported in various LF endemic areas in Egypt at different densities, these include *Cx perexiguus, Cx antennatus, Cx pusillus, Cx sinaiticus, Aedes (Ochlerotatus) caspius, Aedes (Ochlerotatus) detritus* and *Culiseta (Allotheobaldia) longiareolata* (Abdel-Hamid *et al,* 2009; 2011; 2013; El-Naggar *et al,* 2013; Dyab *et al,* 2015).

Application of control measures and mass drug administration to residents of endemic areas in Egypt resulted in the progressive decrease in microfilarial density among residents. Earlier studies indicated that areas with advanced control programs had low endemicity (Fischer *et al,* 2003; Schmaedick *et al,* 2014). Entomological techniques comprise two methods, dissection and PCR-based assays. Dissection has been the gold standard for measuring infection levels in

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mosquitoes; even though it is laborious, tedious and time consuming. Lately, it has become insensitive and inefficient method for detecting filarial parasites in mosquitoes for areas where infection rates have dropped to very low levels following MDA (Goodman *et al*, 2003; Lulitanond *et al*, 2004; WHO, 2013b). Alternatively, PCR–based assays detecting filarial DNA in mosquitoes have provided sensitive and species-specific, and more rapid entomological technique that enables a large number of samples to be processed in a short period (Goodman *et al*, 2003; Bockarie, 2007).

The present results revealed inhibition in 7 pools out of the 70 mosquito pools that were refractory to treatment. It is important to highlight that PCR inhibition with arthropod materials is well documented (Jeyaprakash and Hoy, 2000; Goodman *et al*, 2003; Plichart *et al*, 2006). In fact, inhibition of PCR reactions with DNA extracts from vector mosquitoes is a cause for concern because extracts from the head and thorax are often expected to be enriched for pathogens, whose presence could be masked by the inhibitor (Vezzani *et al*, 2011). Beckmann and Fallon (2012) suggested that an inhibitor that produces false-negative PCR reactions found in the head of *Cx. pipiens* mosquitoes. They noted that DNA pellets commonly had a pink tinge, and reasoned that this pigment might derive from the eyes. Additionally, they found that preparation of template DNA by using a commercially available kit failed to remove the inhibitor, whose molecular identity remains unknown. Nevertheless, the use of commercially available DNA extraction kit have been successfully described in several studies (Rasgon and Scott, 2004; Chambers *et al*, 2009; Plichart and Lemoine, 2013; Schmaedick *et al*, 2014; Dyab *et al*, 2015).

In the present study *W. bancrofti* DNA was not detected in any of the 63 mosquito pools. In Egypt, Dyab *et al.* (2015) carried out a study to detect filarial parasites DNA including *W. bancrofti* in 2,500 female *Culex* mosquitoes, distributed into 100 pools, collected from Assiout governorate. The authors selected the same target region as in the present study, the pWb12 repetitive region, but with different primers using conventional PCR according to Siridewa *et al.* (1996). Results revealed *W. bancrofti* infection in 8% of the mosquito samples. However, other studies performed in Egypt for the detection of *W. bancrofti* DNA in mosquitoes were targeting mostly the Ssp I region. Helmy *et al.* (2004) identified *W. bancrofti* infection in 54.4% of 79 mosquito pools collected from Menoufiya governorate. While Farid *et al.* (2007) carried out a large scale study for molecular xenomonitoring of LF in mosquitoes collected from a total of 402 houses during 1674 nights from Giza and Qualioubiya. Results showed that parasite DNA rates in mosquitoes were greatly reduced to <1% following 5 rounds of MDA. However, the authors found no significant relationship between the infection status of household residents and parasite DNA status of mosquitoes from the same houses.

With the progress of the elimination program in the Nile Delta, the parasite load has reach ultra-low levels, which require larger sample sizes of mosquitoes to detect the presence of infection or even the exposure. Follow up studies are recommended as the prevalence of Culicinae mosquito species predominantly *Cx. Pipiens* the main LF vector may pose a risk of transmission in such areas.

Conclusion

Although there is now wide experience on molecular xenomonitoring, standardization

of the methods will be needed in order to improve the yield of the assays.

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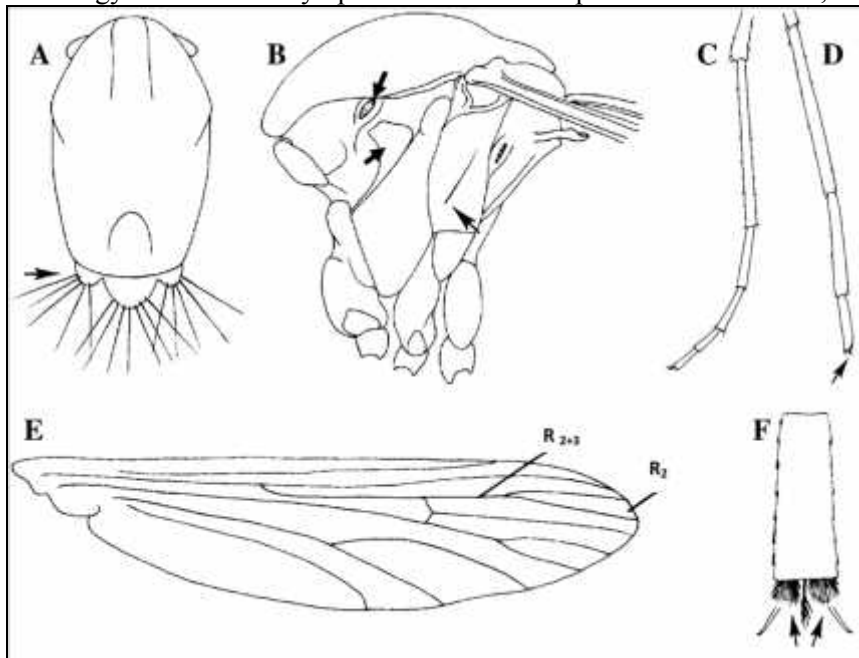

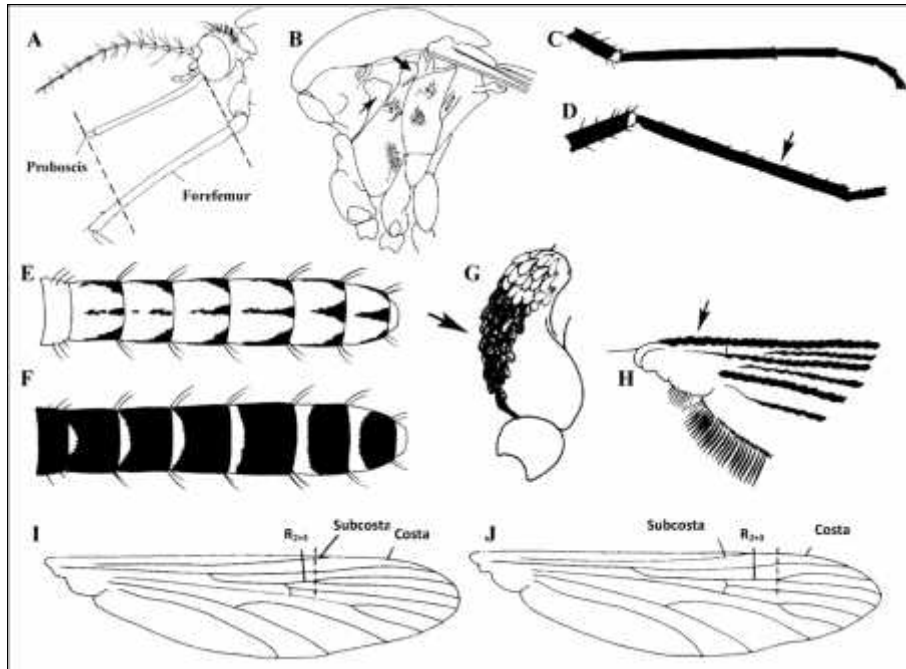Fig. 1: Diagnostic characters of the genus *Culex* (modified from **Harbach, 1985**).

Fig. 2: Diagnostic characters of *Cx. pipiens, Cx. quinquefasciatus* and *Cx. pusillus* (modified from Harbach, 1985).

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