Journal of the Egyptian Society of Parasitology, Vol.43, No.2, August 2013
J. Egypt. Soc. Parasitol., 43(2), 2013: 537 – 546

EFFECTS OF MIDGUT BACTERIA AND TWO PROTEASE INHIBITORS ON THE REPRODUCTIVE POTENTIAL AND MIDGUT ENZYMES OF *CULEX PIPIENS* INFECTED WITH *WUCHERERIA BANCROFTI*

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Abstract

Laboratory investigations were carried out to asses the effect of some proteases inhibitors on the reproductive potential of *Culex pipiens* females resulted from larvae treated with different protease inhibitors. The fecundity and engorgement of symbiotic and aposymbiotic *C. pipiens* females were significantly reduced. The blood meal digestion period increased significantly. On the other hand, enzyme band with molecular weight of 40 KDa which may be cysteine protease was detected in untreated symbiotic and aposymbiotic female midguts.

The results may explain that the absence of this enzyme bands in treated female midguts may be due to the inhibition caused by (E-64) a cysteine protease inhibitor.

Key words: *Culex pipiens, Wuchereria bancrofti*, proteases inhibitors, reproduction.

Introduction

Generally speaking, mosquitoes top all the insect-vectors in transmission of serious zoonotic diseases worldwide. In Egypt, *Cx. pipiens* is widely distributed (Mikhail *et al*, 2009) and the main vector of filariasis which has natural and artificial breeding sites in the endemic and non-endemic villages (Harb *et al,* 1993). Apart from filariasis, Culicini, mainly *Cx. pipiens* transmit Sindbis virus (Wilson, 1991), West Nile Virus, Rift Valley Fever and Dog Heartworm (El-Bahnasawy *et al*, 2012).

Apart from the diseases transmission, mosquitoes can make human life miserable (Morsy, 2012).

The biological impact of protease inhibitors and midgut bacteria on insects has been extensively studied in the laboratory (Awahmukalah and Brooks, 1985; Hogg and Hurd, 1995; Hurd, 2001; Rohani *et al.* 2005; Pais *et al.* 2008). However, only scattered information on effects of proteases inhibitors on protein metabolism and development of the vector host and consequently on the transmission of the filaria is available.

Bacteria exist naturally in the midgut of wild and laboratory-reared mosquitoes (Chao and Wistreich 1960, 1995;

Demaio *et al,* 1996; Pumpuni *et al*, 1996) and Phlebotomines (Schlein *et al*, 1985; Hassan *et al*, 1998). Bacteria occurred in the gut of haematophagous insects may have an important role in epidemiology of human infectious diseases. Such bacteria may interfere with the development of medically important pathogens. For example, high midgut counts of Gram-negative bacteria are known to significantly reduce oocyst numbers in *plasmodium*-infected mosquitoes (Seitz *et al*, 1987, Beier *et al*, 1994; Pumpuni *et al*, 1996).

The present study aimed of evaluation of the role of midgut bacteria of *C. pipiens* in the transmission of *Wuchereria bancrofti* (Human filariasis). Also, to study the effect of proteases inhibitors on protein metabolism and development of the vector host and consequently on the transmission of the filaria.

Materials and Methods

Mosquito rearing: *Culex pipiens* were collected from Abu Rawash City, Giza Governorate, Egypt. They were safely transmitted to the laboratory. 3rd larval instars from the second generation were used in this study. Then placed in plastic cup its diameter was 12 cm and its height was 7 cm containing 250 ml of the proteases inhibitors solution. Control larvae were placed in 250 ml dechlorinated tap water (25 of 3rd instar larvae/cup). The hatching larvae were provided daily with fish food as a diet. This diet was found to be the most preferable food for the larval development and the well female fecundity (Kasap and Demirhan, 1992).

Elimination of bacteria: In order to eliminate *C. pipiens* females midgut bacteria, they were fed on sterile sponge piece soaked in a feeding medium, which composed of 10% sucrose and 0.014 gm of a wide spectrum antibiotic (Tarivid) dissolved in 100 ml. distilled water.

Experimental infection of mosquitoes: The sugar solution was removed 24 hr before the blood meal. *C. pipiens* females were exposed simultaneously to either the upper surface of the hand or on the forearm of the same volunteer between 10 am and midnight. The time of blood feeding coincided with the peak of microfilaraemia recorded in Rocha *et al*. (1991). To reduce discomfort, blood feeding times were limited to 20-30 min.

Dissection of infected mosquitoes**:** The mosquitoes were firstly anaesthetized by chilling and removed from the vial with a pair of fine forceps, and then transferred to a drop of normal sterile saline solution on a microscope slide for dissection under dissecting microscope. The wings and legs were removed using a pair of fine mounting needles and the body transferred to a fresh drop of normal sterile saline solution. To remove the gut from the body one needle was placed on the thorax and the other on the end of the abdomen and gently pulled the digestive tract. The gut was examined by light microscope.

Biological aspects tested: The number of eggs/raft was counted by using a binocular microscope and the mean was calculated. The amount of ingested

blood was determined by weighing 25 females of each treatment immediately before and after blood feeding. The volume of blood ingested was estimated by dividing the weight differences by 1,055 (the approximate density of human blood). The evaluation of blood digestion period was carried out the whole day investigating the amount of blood in mosquito gut.

Electrophoretic enzymes separation: Fifty adult *C. pipiens* females resulted from 3rd instar larvae treated with different protease inhibitors were dissected under dissecting microscope 72 hr post blood meal (PBM) to detect the midgut enzymes using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Gelatin Co-polymerized gel for analysis of proteases according to Laemmli (1970).

Statistical analysis: Statistical analysis of the obtained data was done after Armitage (1974) and Lentner *et al.* (1982), the analysis was revised and graphics were drawn by Excel for windows program version 2 Microsoft office 2010. The obtained data were assessed by calculation of the mean (M), standard deviation (SD) and student t-test.

Results

Effects of protease inhibitors on reproductive potential of symbiotic and aposymbiotic *C. pipiens* females: There was a highly significant decrease ($p>0.05$) of eggs laid by symbiotic females (Tab. 1) resulted from third instar larvae treated with E-64 and a significant decrease ($p>0.05$) of eggs laid by symbiotic females resulted from larvae treated with EDTA, where the fecundity was 167±1.73 and 186.6±3.5 eggs/♀; respectively, compared to 212±13.6 eggs/♀ for the control females. On the other hand, there was a highly significant decrease ($p>0.05$) of eggs laid by aposymbiotic females resulted from larvae treated with E-64 and a significant decrease ($p>0.05$) of eggs laid by symbiotic females resulted from larvae treated with EDTA, where the fecundity was 161.3±1.5 & 183.3± 3.2 eggs/♀; respectively, compared to 212±13.6 eggs/♀ for the control females.

Table 1: Fecundity of symbiotic and aposymbiotic *C. pipiens* females infected with *W. bancrofti* microfilaria resulted from 3rd larval instars treated with different protease inhibitors.

Treatments	No. of females tested	No. of egg laid/female
		Mean ± SD
Control (un-infected)	25	212±13.6
Symbiotic	25	185.6*±3.5
E-64	25	167**±1.73
EDTA	25	186.6*±3.5
Aposymbiotic	25	189.3*±2.1
E-64	25	161.3**±1.5
EDTA	25	183.3*±3.2

Engorgement: There was a significant decrease (p>0.05) of mean weight of blood ingested by symbiotic females resulted from larvae treated with E-64, EDTA and others of untreated (symbiotic) females (Tab. 2). Mean weight of blood ingested was 2.03, 2.01 and 2.01 (mg); respectively, compared to 2.35 (mg) for the control females. On the other hand, there was a significant decrease (p>0.05) of mean weight of blood ingested by aposymbiotic females resulted from larvae treated with E-64, where the mean weight was 2.11 (mg), compared to 2.35 (mg) for the control females.

Table 2: Weight of blood meal ingested by symbiotic and aposymbiotic, *C. pipiens* females infected with *W. bancrofti* microfilaria resulted from 3rd larval instars treated with different protease inhibitors.

Treatments	No. of females tested	Mean weight of mosquito (mg) ± SD		Mean weight of blood ingested (mg)
		Unfed	Engorged	
Control (un-infected)	25	0.88±0.14	3.23±0.3	2.35
Symbiotic	25	0.89±0.02	2.9±0.05	2.01*
E-64	25	0.86±0.03	2.89±0.04	2.03*
EDTA	25	0.86±0.02	2.87±0.05	2.01*
Aposymbiotic	25	0.88±0.014	3.05±0.11	2.17
E-64	25	0.84±0.018	2.95±0.09	2.11*
EDTA	25	0.86±0.03	3±0.16	2.14

Blood meal digestion period: There was a very highly significant increase in the blood meal digestion period of symbiotic females resulted from larvae treated with E-64, EDTA and others of untreated (Tab. 3). The blood meal digestion period was 83.3±1.5 (hrs), 82±1.0 (hrs) and 82±1.0; respectively, compared to 69±0.5 (hrs) for the control females. On the other hand, the results indicated a very highly significant increase in the blood meal digestion period of aposymbiotic females resulted from larvae treated with E-64, a highly significant increase in the blood meal digestion period of aposymbiotic females resulted from larvae treated with EDTA and others of untreated. The blood meal digestion period was 80.5±0.5 (hrs), 78±1.9 (hrs) and 77.8±2.3 (hrs); respectively, compared to 69±0.5 (hrs) for the control females.

Table 3: Digestion period of blood ingested by symbiotic and aposymbiotic, *C. pipiens* females infected with *W. bancrofti* microfilaria, resulted from 3rd larval instars treated with different proteases inhibitors.

Treatments	No. of females tested	Digestion period (hrs) ± SD
Control (un-infected)	25	69±0.5
Symbiotic	25	82***±1
E-64	25	83.3***±1.5
EDTA	25	82***±1
Aposymbiotic	25	77.8**±2.3
E-64	25	80.5***±0.5
EDTA	25	78**±1.9

Electrophoretic enzymes of midgut: The number of midgut enzyme bands (Fig. 1) of the symbiotic *C. pipiens* infected females that separated 72 h (PBM) was 10 and 15 for the females resulted from larvae treated with E-64 and EDTA; respectively. The number of midgut enzyme bands of untreated females recorded was 11 enzyme bands. Also, two major enzyme bands with percentages of 19.9 (M.W. 104 kDa) and 18.5 (M.W. 70 kDa) were among the midgut enzyme bands of untreated females. Three major enzyme bands with percentages of 29.1 (M.W. 104 kDa), 13.2 (M.W. 103 kDa) and 12.0 (M.W. 10 kDa) were detected among the midgut enzyme bands of females resulted from larvae treated with E-64. In addition, three major enzyme bands with percentages of 15.7 (M.W. 104 kDa), 13.2 (M.W. 70 kDa) and 12.2 (M.W. 68 kDa) were detected among the midgut enzyme bands of females resulted from larvae treated with EDTA.

By comparing the electrophoretic enzyme bands of midgut as influenced by protease inhibitors treatment, it appeared that : 1- The number of midgut enzyme bands was variable among untreated and treated females, where it was 10 and 15 bands for E-64 and EDTA-treated females; respectively. 2- The lowest number of enzyme bands (10) was recorded in midguts of (E-64) treated females. 3- Some bands appeared or disappeared according to proteases inhibitor used. 4- The number of enzyme bands with high molecular weights (104-90) was 2 in midguts of untreated females, while it was 3 (104-90) in midguts of E-64 and EDTA-treated females.

On the other hand, the number of midgut enzyme bands of aposymbiotic infected females that separated 72 h (PBM) was 10 and 8 for the females resulted from larvae treated with E-64 and EDTA; respectively. The number of midgut enzyme bands of untreated aposymbiotic females recorded was 8 enzyme bands.

The results obtained showed four major enzyme bands with percentages of 24.0 (M.W. 81 kDa), 23.6 (M.W. 104 kDa), 11.7 (M.W. 13 kDa), and 10.6 (M.W. 84 kDa) among the midgut enzyme bands of untreated aposymbiotic females. The number of major enzyme bands in midgut of proteases inhibitors in treated aposymbiotic females was found to be varied with the protease inhibitors used. Three major enzyme bands with percentages of 19.5 (M.W. 104 kDa), 16.6 (M.W. 84 kDa) and 10.2 (M.W. 86 kDa) were detected among the midgut enzyme bands of aposymbiotic females resulted from larvae treated with E-64. While, five major enzymes among the midgut enzyme bands of EDTA-treated aposymbiotic females were detected. The five major midgut enzyme bands recorded percentages were 20.5 (M.W. 81 kDa), 14.5 (M.W. 18 kDa), 13.6 (M.W. 84 kDa), 13.0 (M.W. 104 kDa) and 12.7 (M.W. 13 kDa).

By comparing the electrophoretic enzyme bands of midgut as influenced

by protease inhibitors treatment, it appeared that: 1- The number of midgut enzyme bands was variable among untreated and treated females, where it was 10 and 8 bands for E-64 and EDTA-treated aposymbiotic females. 2- The lowest number of enzyme bands (8) was recorded in midguts of untreated and EDTA-treated aposymbiotic females. 3- Some bands appeared or disappeared according to proteases inhibitor used. 4- The number of enzyme bands with high molecular weights (104-90) was the same in midguts of untreated and treated aposymbiotic females, it was 2 (104-100), 2 (104-95), and 2 (104-95 kDa) in midguts of E-64, EDTA and untreated females; respectively.

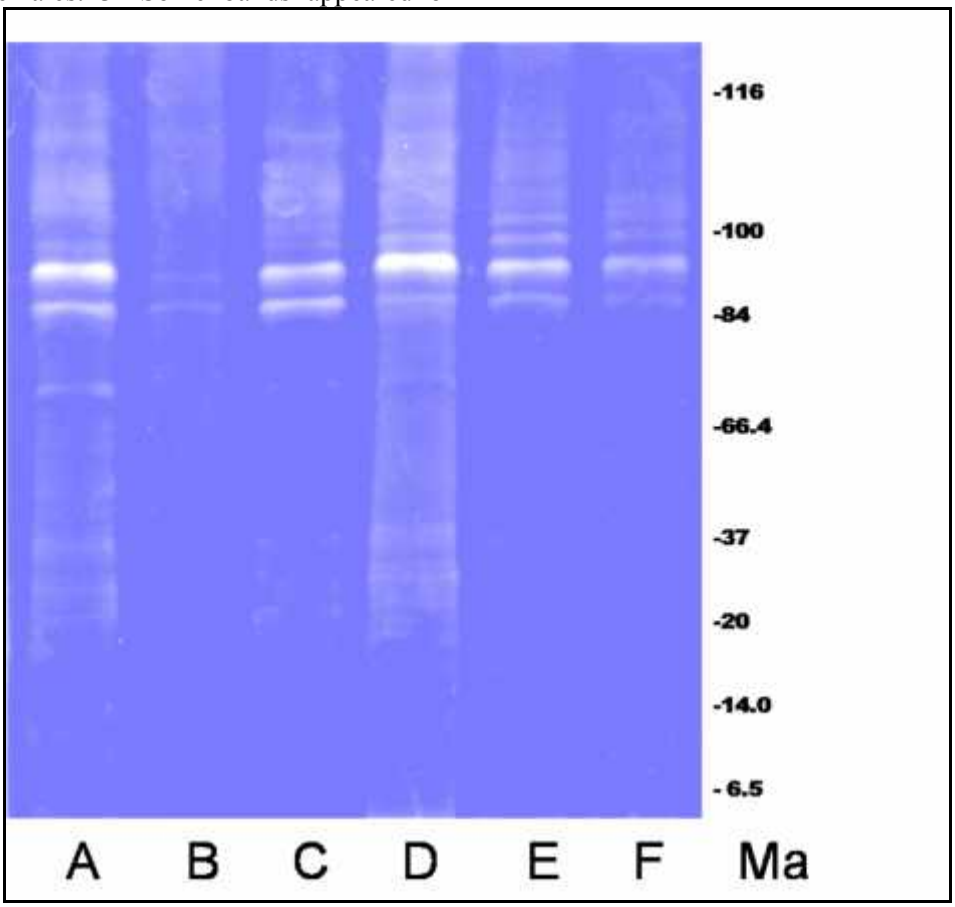

Fig. 1: Zymograms of midgut crude extract of symbiotic and aposymbiotic, *C. pipiens* female midguts treated with different protease inhibitors infected with *W. bancrofti*. (A): Symbiotic females, (B): Symbiotic females treated with E-64, (C): Symbiotic females treated with EDTA, (D): Untreated aposymbiotic females, (E): aposymbiotic females treated with E-64, (F): aposymbiotic females treated with EDTA and (Ma) marker.

Discussion

In the present study, the fecundity of *C. pipiens* females infected with *Wuchereria bancrofti* microfilaria resulted from 3rd larval instars treated with different protease inhibitors was significantly reduced, where the fecundity was167 ±1.73 & 161.3±1.5 eggs/♀ for symbiotic and aposymbiotic *C. pipiens* females resulted from larvae treated with E-64; respectively. The result was 186.6±3.5 & 183.3±3.2 eggs/♀ for symbiotic and aposymbiotic *C. pipiens* females resulted from the larvae treated with EDTA; respectively, compared to 212±13.6 eggs/♀ for the untreated females (control). These data are in a harmony with that of Hogg and Hurd (1995, 1997), Ahmed *et al.* (1999) and Ferguson *et al.* (2003). On the contrary, Hammad (1997) showed that *W. bancrofti* parasites did not interfere significantly with the fecundity.

In the present study, the engorgement of *C. pipiens* females infected with *W. bancrofti* microfilaria resulted from 3rd larval instars treated with different proteases inhibitors was significantly reduced, where the mean weight of blood ingested was 2.03 and 2.11 (mg) for symbiotic and aposymbiotic *C. pipiens* females resulted from larvae treated with E-64; respectively. It was 2.01 & 2.14 (mg) for symbiotic and aposymbiotic *C. pipiens* females resulted from larvae treated with EDTA; respectively, compared to 2.35 (mg) for the untreated females (control). These data in a harmony with those of Hogg and Hurd (1995) who found that infected *Anopheles stephensi* mosquito showed a reduction in mean blood meal size. Contrary, Ahmed *et al.* (1999) showed that *Plasmodium yoelii* parasites did not affect the blood meal size of *Anopheles gambiae* mosquito. However, the interruption of insect reproduction is an important and potent effect for infection.

In the present study, the effect of protease inhibitors on the blood meal digestion period of symbiotic and aposymbiotic females resulted from larvae treated with different protease inhibitors infected with *W. bancrofti* microfilaria increased significantly, where the blood meal digestion period was 83.3±1.5 and 80.5±0.5 (hrs) for symbiotic and aposymbiotic *C. pipiens* females resulted from larvae treated with E-64; respectively. It was 82±1 and 78±1.9 (hrs) for symbiotic and aposymbiotic *C. pipiens* females resulted from larvae treated with EDTA; respectively, compared to 69±0.5 (hrs) for the untreated females (control). These results may be comparable with those obtained by Rohani *et al.* (2005) who demonstrated that the rate of digestion for blood meals was slower for both *Aedes aegypti* and *Ae. albopictus* mosquitoes when fed with dengue virus infected blood.

In the present study, a cysteine protease band with molecular weight of 40 kDa was detected in midgut of pea aphid *Acyrthosiphon pisum* by Cristofoletti *et al*. (2003). Moreover, they observed that this enzyme inhibited by E-64 and activated by EDTA+cysteine. The present results of electrophoretic enzymes in midguts of symbiotic and

aposymbiotic, *C. pipiens* females 3 days post infected blood meal may be comparable to the previous findings, where enzyme band of a molecular weight of 40 kDa was detected among enzyme bands of untreated symbiotic and aposymiotic, *C. pipiens* midguts.

Conclusion

The Culicine situation necessitates a worldwide vector control program to minimize the Egyptian lymphatic filariasis transmission.

It has been now on going successful but, relatively slow Egyptian prophylactic and eradicative filariasis health campaigns, more stress on educational media programs is required in remote endemic areas.

The values of biological aspects tested were not greatly influenced with the elimination of midgut bacteria of *C. pipiens* females as compared with normal females (symbiotic ones). While, the absence of enzyme bands with molecular weight of 40 kDa which believed to be a cysteine protease in treated females which may be played a great role on inhibition of the parasite transmission.

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