Response of *Anopheles stephensi* to Deltamethrin in Lahore District, Pakistan

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In the present study, both males and females of *Anopheles stephensi* Liston were sampled from three different localities of Lahore district of Punjab, Pakistan. (Jaman, Mohlanwal and Noorpur Bhatta A1). Bioassays against 0.05 % deltamethrin were performed to evaluate the level of resistance in *An. stephensi*. All three populations were found susceptible to the WHO recommended dose of deltamethrin. Mortality rates were significantly higher in males than females. Biochemical tests were performed to measure the esterase activity, both in males and females of *An. stephensi*. All three populations displayed a large variation in the levels of both esterases A and B. Females were found to have significantly higher activity of esterase A and esterase B than males. Only one band (A1) was observed for esterase A in all three populations. For esterase B two bands (B1 and B3) were observed in Mohlanwal and Noorpur Bhatta A1. However, in Jaman a third bands (B2) of moderate intensity was also present between B1 and B3. Results of the present study suggested that present variations and high level of esterases in populations of *An. stephensi* in district Lahore are not associated with resistance against deltamethrin.

Key Words: Esterases, *Anopheles stephensi*, Resistance, Pyrethroids.

INTRODUCTION

Mosquitoes of genus *Anopheles* not only cause a severe biting nuisance, primarily in the summer months in Pakistan but also are a serious threat to public health. There are over 577 species of this genus throughout the world out of which 77 species are confirmed vectors for malaria. In Pakistan about 24 species of *Anopheles* are reported and among them two are major malarial vector species *An. culicifacies* Giles and *An. stephensi*. Two new species *An. fluviatilis* James and *An. annularis* Van der Wulp are suspected to be vectors for malaria in Baluchistan province¹.

Resistance to pyrethroids has been reported in major groups of insects including mosquitoes (*i.e.*, *Culex* and *Anopheles*) from South America, Sudan, Sri Lanka, Nigeria, Burkina Faso, Egypt, Guatemala, USA, Turkey and Syria²⁻⁴. Many of the resistant insects serve as vectors of emerging diseases. Insecticide resistance is expected to directly affect the re-emergence of vector born diseases and it is expected to threaten disease control activities⁵⁻⁷. The resistance mechanisms involve esterases, glutathion S-transferases and/or monooxygenases⁸. However, the most common mechanism of insecticide resistance in insects is modified level or activities of esterases that metabolize a wide range of insecticides^{9,10}. Enhanced esterase activity has been detected in a number of

insecticide resistant insects^{11,12}. The esterase-based resistance mechanism has also been detected in *An. stephensi*^{13,14}.

Interest has been generated in biochemical tests for detecting and monitoring insecticide resistance that are more specific than traditional bioassays. Detection of specific resistance mechanisms in individual insect and estimation of resistance genes in a population is the major objective of such tests¹⁵. Most biochemical approaches developed thus far, aim at the detection or measurement of enzymatic reaction in homogenates of a single insect and the use of electrophoretic analysis, filter paper tests or micro titer plate assays. Many biochemical tests are concerned with the detection of organophosphate and carbamate resistance due to reduced sensitivity of acetylcholinesterase¹⁶⁻¹⁸ or increased detoxification by highly active esterases in mosquitoes^{19,20}.

In Pakistan (Punjab), malathion (organophosphate) was used to control mosquitoes before 1991. However after 1991 malathion was replaced gradually by deltamethrin (synthetic pyrethroid), due to emergence of resistance in *An. stephensi* against malathion, as reported from some areas of Pakistan (Unpublished Surveillance data of communicable disease control programme of Punjab Health Department). Deltamethrin produced 100 % mortality in *An. culicifacies* and *An. stephensi* and up to year 2002 no resistance has been reported against deltamethrin in country. The aim of present study was to

investigate the following concerns:

- (1) Are there any deltamethrin resistant *An. stephensi* populations in the Lahore district?
- (2) Is there any difference in the levels of esterases in different populations of *An. stephensi*?
- (3) Is there any difference in the quantity of esterases in male and female specimens of a population?
- (4) How many types of esterases are present in different populations of *An. stephensi* in Lahore district?
- (5) Is there any correlation between levels of esterases and previous exposure of insecticides?

EXPERIMENTAL

Selection sites: On the basis of surveillance and operational data from the communicable disease control programme of the health department district Lahore. We have selected three localities (*i.e.*, Jaman, Mohlanwal and Noorpur Bhatta A1) from rural areas for sampling. At present *An. stephensi* is found only in rural areas of the district. Moreover mosquito populations at these localities have a history of exposure to pyrethroids, especially deltamethrin used in the public health program¹.

Sampling: During this study, *An. stephensi* were collected from living rooms, stores and animal sheds using an aspirator (WHO, 1992). Total sample size was 1200 (400 individual from each locality). Identification was made using the pictorial key developed by the National Institute of Malaria Research and Training, Lahore, a sub-center of the National Institute of Health, Islamabad.

Mosquitoes were divided into two groups. One group of female mosquitoes was subjected to adult bioassay tests and the second was stored at -20 °C for further biochemical estimation of esterases and polyacrylamide gel electrophoresis.

Adult bioassays: Blood-fed females ($n = 25$) and males ($n = 25$) of *An. stephensi* were used for the bioassays²¹. Mosquitoes collected from each locality were exposed for 1 h to 5 % deltamethrin impregnated papers using WHO standard exposure tubes³ and then transferred to holding tubes (tubes without impregnated papers). Three replicate tests were performed. Mortality of mosquitoes on control and treated papers was recorded after 24 h post exposure.

Biochemical estimation of esterases: Esterase activity was measured in homogenates of single adult mosquitoes of almost same size ($n = 25$). Each adult was homogenized in 50 μ L of 40 mM phosphate buffer, pH 7.0, containing 0.01 % (w/v) of Triton X-100¹⁹. The homogenate was centrifuged at 13000 rpm for 5 min. Supernatant was used in assay with α -naphthyl acetate (substrate A) and β -naphthyl acetate (substrate B). The reaction mixture contained 30 μ L homogenate, 60 μ L substrate solution (0.1 M), 100 μ L SDS (5 %) and 1440 μ L phosphate buffer. After an incubation period of 0.5 h at 37 °C, optical density was recorded at a wave length of 620 nm for α -naphthyl acetate and at 545 nm for β -naphthyl acetate. The amount of esterase present was calculated by converting the optical density values into ng/mL of esterase using standard curves, which were prepared with different concentrations of α - and β -naphthol (Fig. 1a and b).

Polyacrylamide Gel electrophoresis: Polyacrylamide gel electrophoresis was performed to separate different types of

α - and β -esterases in different populations of *An. stephensi*. Only females of equal size were used for this purpose. Two concentrations of gel were used in polyacrylamide gel electrophoresis *i.e.*, separating gel and stacking gel. Clean gel plates were clamped firmly after placing spacers on the two sides of the gel plates. The plates were surrounded by gaskets on three sides to prevent any leakage. The separating gel (8 %) was prepared by mixing 9.7 mL distilled water, 5.3 mL acrylamide and bisacrylamide (30 %), 5 mL tris buffer (pH 8.8), 0.2 mL SDS (10 %), 0.2 mL ammonium persulphate (10 %) and 0.012 mL tetramethylethylenediamine. This solution was poured into the space between the two glass plates. The stacking gel was prepared by mixing 3.4 mL distilled water, 830 μ L acrylamide and bisacrylamide (30 %), 630 μ L tris buffer (pH 6.8), 50 μ L SDS (10 %), 50 μ L ammonium per-sulphate (10 %) and 5 μ L tetramethyl ethylenediamine.

Fig. 1. Standard curve for α -naphthol (a) and β -naphthol (b)

The stacking gel was poured over the separating gel and a comb was inserted to make the wells. From each locality 15 female mosquitoes of same size were randomly selected and crushed in 300 μ L phosphate buffer. The homogenate was centrifuged at 13000 rpm for 5 min. From the supernatant 100 μ L of sample was mixed with 100 μ L of α - or β -naphthyl acetate and then incubated for 35 min at 37 °C. Then 150 μ L sample buffer was also added to the sample and heat shock

was given to the sample for 5 min. Gel plates were adjusted in a vertical position to the gel apparatus. An electrode buffer was added in both the upper and lower tanks of the gel apparatus. The gel was run at 150 v with continuous cooling at 4 °C until the dye reached the lower plates. The gel was stained with fast blue RR stain for ten minutes. De-staining was done by methanol to remove the extra stain. When bands of esterases became clear, the gel pattern was studied.

Statistical analysis: Fisher's exact test was used to compare the mortality rate of mosquitoes at different time intervals. Paired *t*-test was used to compare the mortality in male and female mosquitoes.

Levels of esterases in different populations of *An. stephensi* were compared by using ANOVA. Paired *t*-test was also used to compare the level of esterases in male and female mosquitoes using Minitab Software (version 13.3)

RESULTS AND DISCUSSION

Adult bioassays: Mosquitoes from all three populations were found susceptible using the WHO recommended dose of deltamethrin (*i.e.*, 5 %). After 24 h exposure, 100 % mortality was recorded. The mortality response of *An. stephensi* was highest between 3 to 12 h after insecticide exposure in all populations (Fig. 2). In all three populations mortality over time was significantly higher in males than females ($t = 4.99$; $P < 0.001$).

Fig. 2. Percent mortality of *An. stephensi* after 1 h exposure to deltamethrin (combined for three populations); Note: Bars in the figure with different superscripts are showing significant difference in percent mortality

Biochemical estimation of esterases: One-way ANOVA, performed to compare the levels of esterases in three different populations of *An. stephensi*, showed statistically significant variation ($F_{2, 72} = 4.29$; $P < 0.05$). The highest level of esterase activity was recorded in the Jaman population while the lowest was seen in the population from Noorpur Bhatta A1. It was observed that the mosquito populations from Jaman had high esterase activity against β -naphthyl acetate (Substrate B) while the other two populations (*i.e.*, Mohlanwal and Noorpur Bhatta A1) had high esterase activity against α -naphthyl acetate (Substrate A). Moreover, all three populations displayed a large variation in the levels of both esterases A and B. Females were found to have significantly higher activity of esterase A ($t = 4.99$; $P < 0.001$) and esterase B ($t = 5.02$; $P < 0.001$) than males.

Polyacrylamide gel electrophoresis: Results of polyacrylamide gel electrophoresis indicated differential profiles regarding

the mobility, intensity and number of bands for esterases A and esterases B. Only one band (A1) was observed for esterase A in all three populations (Fig. 3a). Differential band patterns were observed for esterases B in populations of three localities (Fig. 3b). Two bands, each of similar nature were observed in all the three populations. One slow moving band of high intensity occupied the upper part of the gel in the samples of all localities and was labeled as B1 while the narrow band (B3) of less intensity was present in the bottom of gels of all samples. In the population of Jaman a third bands (B2) of moderate intensity was present between B1 and B3. This band was absent in the population of Noorpur Bhatta A1 and Mohlanwal.

Fig. 3. Polyacrylamide gel of *Anopheles stephensi* showing esterase A (a) and esterases B; Note: A = Jaman, B = Mohlanwal, C = Noorpur Bhatta A1

Pyrethroid resistance is emerging despite early optimism that because of its rapid toxicological action this newest large class of insecticides would not produce resistance. Several studies have reported the development of resistance against pyrethroids in mosquitoes in different parts of the world such as Middle East, South America, Sudan, Sri Lanka, Nigeria, Burkina Faso, Egypt, Guatemala, USA, Turkey and Syria^{2,3}. Results obtained during bioassays in the present study revealed that individuals of all three populations were susceptible to the WHO recommended dose (5 %) of deltamethrin. The mortality was higher between 3rd and 12th hour and no knockdown resistance was found. Present results were in contrast with the findings of Enayati *et al.*²² who reported knockdown resistance in *An. stephensi* from Dubai. Ganesh *et al.*²³ also reported tolerance to deltamethrin in larvae of *An. stephensi* from Mysore. Though a small proportion of the population from Jaman and Mohlanwal had increased level of esterase A and

esterase B, the populations were susceptible during bioassays. Ocampo and Wesson²⁴ also reported that a small proportion of *An. pseudopuntipennis* from Colombia, having high levels of esterases were susceptible during bioassay against pyrethroids.

Significant variation was recorded in esterase levels among populations and within the tested populations. Pasteur *et al.*¹⁵ also observed variation in quantity of esterases in field-collected mosquitoes. The population from Jaman showed more variation in enzyme activity to hydrolyze the β -naphthyl acetate (Substrate B) compared to populations at Mohlanwal and Noorpur Bhatta A1. Variations of esterases in these three populations may be due to exposure of individuals to organophosphate compounds. All localities have agricultural fields and organophosphates are being used for agriculture in these areas. Greater variation in quantity of esterases B in Jaman than the other two localities may be related to the fact that the area used for agriculture in this locality is much greater than the other two localities. Thus the use of organophosphate compounds is much heavier in this locality.

According to Roush²⁵ esterases are important in resistance to organophosphate insecticides in mosquitoes and occasionally they play role against pyrethroids. α -Naphthyl acetate showed high activity with mosquito homogenates of Noorpur Bhatta A1 and Mohlanwal, indicating the predominance of esterases A in these populations. The increased levels of esterase A have no contribution toward the development of resistance against deltamethrin in these populations of *An. stephensi* as demonstrated by the lack of resistance detected in WHO bioassays. Organophosphate compounds are still in use for agriculture practice might be the reason of increased level of esterases A. However, the mosquito homogenates of Jaman populations hydrolyzed the beta naphthyl acetate faster than α -naphthyl acetate which is an indication for the dominance of esterases B. Ganesh *et al.*²³ have found significant correlation between increased esterases B and development of tolerance against deltamethrin in the larvae of *An. stephensi*. However, in Jaman esterase B may be related to greater exposure of individuals to the organophosphate because population was also found susceptible to deltamethrin during bioassays. Mouches *et al.*²⁶ and Beyssat *et al.*²⁷ have also reported that increased esterases B are responsible for organophosphate compounds resistance in *An. stephensi* and *Cx. quinquefasciatus*. Tahir *et al.*²⁸ has also reported an increase in esterases B and esterases A in different populations of *Cx. quinquefasciatus* from the district of Lahore not related with pyrethroid resistance. Results of the present study are also in contrast with the result of Bull *et al.*²⁹ who found greater esterases B in pyrethroid-resistant populations. This could be interpreted to mean that this esterase is involved in pyrethroid resistance but according to present study esterase A is not involved in pyrethroid resistance because those populations, which have greater exposure to pyrethroid showed high values of optical densities with α -naphthyl acetate.

Higher levels of esterases A and esterases B were found in female *An. stephensi* than males in the present study. This difference level in males and females might be associated with the longevity of females compared to males. During their life span, females have greater exposure to insecticides, which may

result in enhanced level of detoxifying enzyme in comparison to males. These results are contrary to Krafsur and Ernst³⁰ who reported high levels of only esterases B in female but not A in horn flies. Some studies have shown that both esterases A and B are high in females^{19,20}.

During the present study differential profiles for esterases A and esterases B regarding mobility, intensity and number of bands was noted. A differential profile of esterases A and esterases B has also been reported by Ganesh *et al.*²³ in *An. stephensi* and *An. culicifacies*. Only one kind of band was observed in all three sampled populations for esterases A. Dubash *et al.*³¹ has reported four electrophoretic variants of esterases A in laboratory strain of *An. culicifacies*. Bisset *et al.*³² reported two types of esterases A in *Cx. quinquefasciatus* from Cuba and Tahir *et al.*²⁸ has reported one type of esterases A in *Cx. quinquefasciatus* from Lahore Pakistan.

For esterases B, three bands (B1, B2 and B3) in Jaman and two bands in Noorpur Bhatta A1 and Mohlanwal populations were observed. A third band (B2) was present only in Jaman and was absent in Noorpur Bhatta A1 and Mohlanwal. Beyssat *et al.*²⁷ has reported one band of esterases B in *An. stephensi*, Bisset³³ has reported three types of esterases B in *Cx. quinquefasciatus* from Cuba whereas Tahir *et al.*²⁸ has reported three types of esterases B from Lahore, Pakistan.

From the present studies it is concluded that present variations and high level of esterases in populations of *An. stephensi* in district Lahore are not producing resistance against deltamethrin. Males have lower levels of esterases compared with females. There is only one type of esterase A in all populations while 2-3 types of esterases B are present.

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