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Susceptibility/resistance of selected insecticides in Anopheles mosquitoes of district Mirpur Khas, Sindh, Pakistan

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Abstract
Vector control is an effective way of reducing malaria transmission. The main vector control methods include the use of insecticide-treated bed nets and indoor residual spraying (IRS) and larviciding. Pyrethroids represent the only class of insecticides approved for treating bed nets and currently used in malaria control strategy. However, insecticide resistance, in particular pyrethroid cross-resistance, is a challenge facing malaria vector control in district Mirpur Khas.

Anopheles mosquitoes were collected from four malaria surveillance sites i.e Goth Limoon, Goth Bhoorji, Goth Ahmed Khan, and Goth Kamal Khan Kapri of district Mirpur Khas Pakistan. The mosquitoes were collected from field and identified using morphological keys. Field collected adult female mosquitoes *Anopheles subpictus* were tested for susceptibility using standard WHO protocols, susceptibility test kits and insecticide treated papers. Anopheles subpictus were exposed to various diagnostic doses of three pyrethroids (Deltamethrin, Lambda-cyhalothrin, and Permethrin), one organophosphate (malathion), and one chlorinated hydrocarbon (DDT), showed a range of mortalities in various localities of district Mirpur Khas. *Anopheles subpictus* was resistant to four out of five insecticides in all four localities.

Results of test done on *Anopheles subpictus*, against diagnostic dose of 4 % DDT shown percentage mortality ranged from 50.44 % to 100 %. *Anopheles subpictus*, shown no resistance against 5 % Malathion with mortality rate of 100 %.Test results against 0.05 % Deltamethrin diagnostic dose showed resistance in *Anopheles subpictus* with percentage mortality ranged from 64.75 % to 100 %. Wild caught female *Anopheles subpictus* showed percentage mortality ranged from 56.89 % to 77.40% against 0.05 % Lambdacyhalothrin, while against 0.75 % Permethrin the species were resistant with percentage mortality ranged from 54.78 % to 67.21 % in all four localities.

This current survey of insecticide resistance in Anopheles species provides baseline information for monitoring resistance in district Mirpur Khas and highlights the need for routine resistance surveillance as an integral part of the vector control programme.

Keywords: insecticides, anopheles mosquitoes, malaria transmission, district Mirpur Khas

Introduction
Malaria is a mosquito-borne parasitic disease that is common in the world's poorest countries. It is preventable and treatable, yet it still kills millions of people every year [1]. Approximately half of the world's population is at risk of malaria and most malaria cases and deaths occur in sub-Saharan Africa. However, Asia, Latin America, and to a lesser extent the Middle East and parts of Europe are also affected. In 2011, 99 countries had ongoing malaria transmission. According to the latest estimates, there were about 219 million cases of malaria in 2010 and an estimated 660 000 deaths. Most deaths occur among children living in Africa where a child dies every minute from malaria [2]. In the Eastern Mediterranean Region, Pakistan, Somalia, Sudan and Yemen whereas in the South-East Asia Region, Bangladesh, India, Indonesia and Myanmar have persistently high malaria burden. In 2010, four countries accounted for 97% of the confirmed cases which were Sudan (58%), Pakistan (22%), Yemen (10%) and Afghanistan (6%) [3]. Malaria is the second most prevalent and devastating disease in Pakistan. Malaria has a tendency for epidemic outbreaks over larger area, particularly in Balochistan, NWFP and Sindh province. However, the disease is now emerging as a prominent health problem in FATA particularly along the international border with Iran and Afghanistan. In 2011, the total number of confirmed malaria cases in Pakistan (public sector), reported from all the districts were 319,592 [4].

About 380 species of Anopheles occur around the world. Some 60 species are sufficiently attracted to humans to act as vectors of malaria [5]. In Pakistan, out of total 24 Anophelines there are two major vector species named *Anopheles culicifacies* and *Anopheles subpictus.* Recently two new species *An. fluviatilis* and *An. annularis* have been identified from Baluchistan province. Previously these two species have been considered confirmed malaria vectors in Iran and Afghanistan. However, thier role as malaria vector need to be confirmed through systematic operational research [4].

Vector control is an important part of the global malaria control strategy. Vector control is highly dependent on the use of pyrethroids, which are the only class of insecticides currently recommended for ITNs or LLINs. In recent years, mosquito resistance to pyrethroids has emerged in many countries. In some areas, resistance to all four classes of insecticides used for public health has been detected. Sub-Saharan Africa and India are characterized by high levels of malaria transmission and widespread reports of insecticide resistance. The development of new insecticides for use on bed nets is a particular priority. Detection of insecticide resistance should be an essential component of all national malaria control efforts to ensure that the most effective vector control methods are being used. The choice of insecticide for IRS should always be informed by recent, local data on the susceptibility target vectors [2].

In world DDT (Dichloro- diphenyl trichloroethane) was first introduced for mosquito control in 1946 and in 1947 the first case of DDT resistance occurred in mosquitoes. Since then more than 100 mosquito species are reported as resistant to one or more insecticide, and more than 50 of these are anophelines. The development of pyrethroid resistance in *An. gambiae* is reported by the WHO and other organizations on the use of pyrethroid-impregnated bed nets for malaria control. The West African has higher levels of resistance than that in east Africa [6].

Malaria vectors have also acquired widespread resistance to many of the currently used insecticides, including synthetic pyrethroids. Hence, there is an urgent need to develop alternative insecticides for effective management of insecticide resistance in malaria vectors [7].

The two primary malaria vectors in Pakistan *An. subpictus and An. Culicifacies* have developed resistance to insecticide of chlorinated hydrocarbon group such as DDT and dieldrin in neighboring countries such as Iran, Afghanistan, Iraq, Saudi Arabia, India and these species developed resistance to malathion in Iran.. In Pakistan resistance to previously used organo-chlorides (DDT, dieldrin), carbamates (propoxur) and organophosphates (malathion, fenitrothion) has been well documented. The Malaria Control Programme has used pyrethroids for both indoor residual spraying and long-lasting insecticide-treated nets since 1992, and the efficacy of this group of insecticides needs further validation [1].

In order to ensure a timely and coordinated global response to the threat of insecticide resistance, WHO has worked with a wide range of stakeholders to develop the Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM), which was released in May 2012. The GPIRM puts forward a five-pillar strategy calling on the global malaria community to plan and implement insecticide resistance management strategies in malaria-endemic countries; ensure proper and timely entomological and resistance monitoring, and effective data management; develop new and innovative vector control tools; fill gaps in knowledge on mechanisms of insecticide resistance and the impact of current insecticide resistance management approaches; and ensure that enabling mechanisms (advocacy as well as human and financial resources) are in place [2]. Keeping in mind the Global Plan for Insecticide Resistance Management in malaria vectors (GPIRM), timely entomological and resistance monitoring will help to plan effective malaria control programme, this study is based on that plan.

Resistance/Susceptibility status of Anopheles mosquitoes has not yet established in Mirpur Khas district so this study will provide baseline data on Resistance/Susceptibility status of Anopheles mosquitoes against different groups of insecticides.

Materials and Methods

Materials

CDC sweeper, Stereo microscope, Paper cups, WHO test kit, Insecticide impregnated papers

Method

The World Health Organization standard procedure was used for the susceptibility/Resistance status. 20–50 female mosquitoes were aspirated into each holding tube through the filling hole in the slide. Then slide unit was closed and the holding tubes set in an upright position for one hour. At the end of holding time, damaged insects were removed. Each of the 4 red-dotted exposure tubes was lined with a sheet of insecticide-impregnated paper, while the 2 yellow-dotted control exposure tubes were lined with oil-impregnated papers; each was fastened into position with a copper spring-wire clip. The empty exposure tubes were attached to the vacant position on the slides and with the slide unit open the mosquitoes were blown gently into the exposure tubes. When all the mosquitoes were in the exposure tubes, the slide unit was closed and the holding tubes were detached and set to one side. Mosquitoes were kept in the exposure tubes, which were set in a vertical position with the mesh-screen end uppermost, for a period of 1 hour (60 minutes). At the end of the 1-hour exposure period, the mosquitoes were transferred back to the holding. The exposure tubes were detached from the slide units. A pad of a cotton-wool soaked in sugar water was placed on the mesh-screen end of the holding tubes. Mosquitoes were maintained in the holding tubes for 24 hours (the recovery period). During this time, the holding tubes were kept in a shady place to maintain extremes of temperature. Temperature was recorded as 23-27 ºC, and humidity was 70-85 % during the recovery period. At the end of recovery period (i.e. 24 hours post-exposure), the number of dead mosquitoes were counted and recorded on susceptibility test forms.

Interpretation of susceptibility test results

- Mortality in the range 98–100 % indicates susceptibility.
- A mortality of less than 98% is suggestive of the existence of resistance and further investigation is needed.
- If the observed mortality is between 90 % and 97 %, the presence of resistant genes in the vector population must be confirmed. The confirmation of resistance may be obtained by performing additional bioassay tests with the same insecticide on the same population or on the progeny

of any surviving mosquitoes (reared under insectary conditions) and/or by conducting molecular assays for known resistance mechanisms. If at least two additional tests consistently show mortality below 98 %, then resistance is confirmed.

- If mortality is less than 90 %, confirmation of the existence of resistant genes in the test population with additional bioassays may not be necessary [30].

Data Entry: Data was analyzed by using SPSS_16 software to find the chi-square to check the population status either it is homogeneous or heterogeneous and p. value to find the level of significance of tests from different localities.

Plan of Analysis

The Percentage mortality of test sample was calculated by summing the number of dead mosquitoes across all four exposure replicates and expressing this as a percentage of the total number of exposed mosquitoes:

$$\text{Observed mortality} = \frac{\text{Total number of dead mosquitoes} \times 100}{\text{Total sample size}}$$

Results

Two sentinel sites (Goth Limoon, Goth Bhoorji) in district Mirpur, Sindh Pakistan were selected with high density of vector population. The results showed that at Goth Limoon out of 114 exposed mosquitoes 12 mosquitoes was dead after one hour exposure period and after 24 hour holding period 86 was dead out of 114 exposed mosquitoes. Percentage mortality after one hour was 11.76 % and after 24 hour percentage mortality became 75.44 % with chi-square value 1.52 and P-value 0.823 against DDT and interpreted as resistant ®. Temperature and humidity was 3 ^{0}C and 72 % respectively while against Malathion out of 113 total expose mosquitoes 109 was dead and after 24 h exposure period all dead. Percentage mortality after one hour and 24 hour was calculated as 96.46 % and 100 % respectively with chi-square value 2.688 and P-value 0.611 so interpreted as susceptible (S). In case of Deltamethrin after one hour exposure period 60 mosquitoes were survived out of 118 and at the end of holding period only 13 mosquitoes were survived out of 118. Percentage mortality after one hour and 24 h were calculated as 49.15 % and 88.98 % respectively with chi-square value 1.10 and P-value. 894. So we interpreted as resistant ®. The test result of Lambdacyhalothrin 0.05 % showed that out of 115 exposed mosquitoes 23 were dead after one hour and after holding period 89 were dead out of 115 total mosquitoes. Percentage mortality after exposure and holding period were 20 % and 77.40 % respectively with chi-square value 1.435 and P-value 0.838 so results interpreted as resistant to that insecticide ® while expose to permethrin 0.75 % 25 dead out of 115 total exposed mosquitoes after one hour and after 24 hour 75 dead out of this total. Percentage mortality was calculated as 21.73 % after one hour and 65.21 % after 24 h with chi-square value 0.375 with P-value 0.984 so results concluded that mosquitoes were resistant to that insecticide. Geographical comparison of percentage mortality against tested insecticides given in figure-1.

Graphical comparison of % mortality of tested insecticides against Anopheles specie Goth Limoon

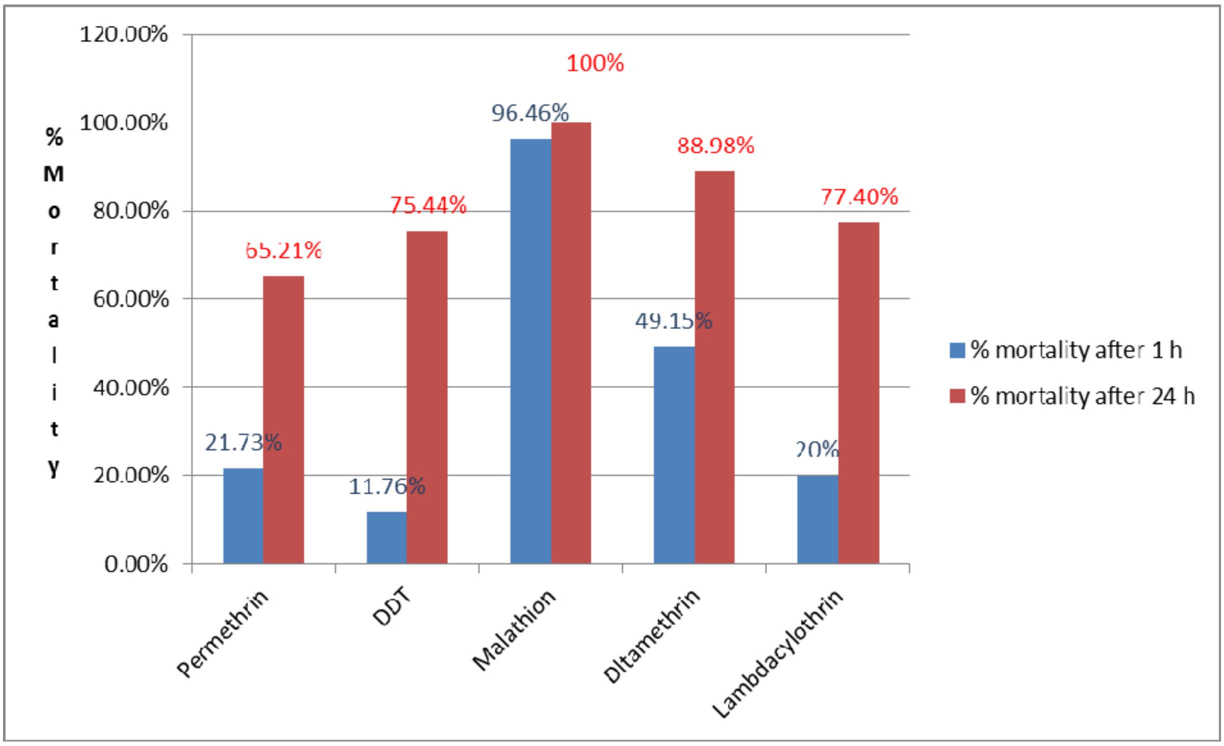

Fig 1

The results recorded at Goth Bhoorji Mirpur Sindh Pakistan showed that exposure to 4% DDT out of 116 total expose mosquitoes 94 were found dead after one hour exposure and after 24 hour holding period all mosquitoes were found dead. Percentage mortality was calculated as 81.03% and 100% after one hour and 24 hour respectively with P-value 0.927 and chi-square value 0.883 while exposure to 5% Malathion out of 118 total exposed mosquitoes 107 were found dead after one hour and after 24 hour all 118 mosquitoes were dead. Percentage mortality after exposure period and hold period were calculated as 98.68% and 100 % respectively with P-value 0.871 and chi-square value 0.241. Results were interpreted as Resistant to specific insecticide ® while when mosquitoes that was 119 exposed to recommended dose of 0.05% Deltamethrin in first hour of exposure period only 86 specimens were found dead and after 24 hour holding period 100% mortality were observed with P-value 0.933 and chi-square value 0.841. Results were interpreted as mosquitoes Susceptible (S) to Malathion in that locality. In the exposure of 0.05% Lambdacyhalothrin 40 and 80 mosquitoes were found dead out of 118 mosquitoes in exposure period and holding period respectively. Percentage mortality was 33.89% and 67.79% after exposure period and holding period respectively. Data analysis provided us P-value 0.729 and chi-square value 2.036 while 0.75% permethrin results showed that 37 mosquitoes were observed dead in exposure period and after holding period mortality ranged to 77 out of 114 exposed mosquitoes. Percentage mortality was 32.45% and 67.54% in exposure and holding period respectively. P-value and Chi-square value was 0.729 and 0.036 respectively. Geographical comparison of percentage mortality after holding period of all tested insecticides showed in figure-2

Graphical comparison of % mortality of tested insecticides against Anopheles specie Goth Bhoorji

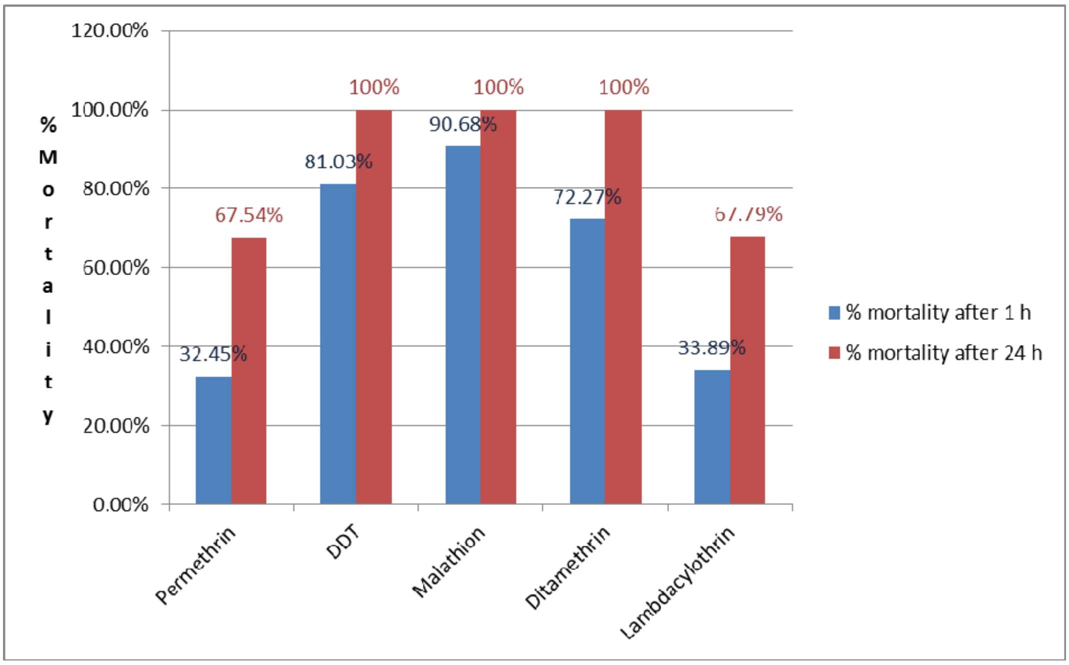

Fig 2

Discussion

Anopheles subpictus was exposed to various diagnostic doses of three pyrethroids (deltamethrin, lambda-cyhalothrin, and permethrin), one organophosphate (Malathion), and one chlorinated hydrocarbon (DDT), showed a range of mortalities in various localities of district Mirpur Khas. *Anopheles subpictus* was resistant to all five insecticides.

Results of test done on *Anopheles subpictus,* against diagnostic dose of 4% DDT which belongs to chlorinated hydrocarbon showed resistance in all four localities with percentage mortality ranged from 64% to 100%. Malathion belongs to group of organophosphate. The results of test done on, *Anopheles subpictus*, against 5% Malathion, showed complete mortality of 100% and *Anopheles subpictus* is susceptible to 5% malathion. Next three insecticides belong to pyrethroid group. Test results against 0.05% deltamethrin diagnostic dose showed complete resistance in all four localities in *Anopheles subpictus* with percentage mortality ranged from 71% to 100%. Wild caught female *Anopheles subpictus* showed percentage mortality ranged from 68% to 83% and show resistance at all two localities against 0.05% lambda cyhalothrin while against 0.75% permethrin *Anopheles subpictus* were resistant with percentage mortality ranged from 63% to 77% in all two localities.

Chi-square tests were performed to compare populations collected from different locations for heterogeneity. At least five replicates of each were made from nearly all locations. In the late 1970s, the Sindh province faced malaria control failure due to undetected resistance to DDT and malathion, which were in use at that time. Due to this history, the judicious use of pesticides, supported by appropriate insecticide resistance monitoring in Sindh has assumed top priority, particularly for pyrethroids, which are presently not only used for public health, but are also used in large quantities for agricultural purposes. Such a situation calls for intensified monitoring and surveillance of resistance in insect vectors.

The results of the present study were compared with the results of previous studies in which resistance to DDT and malathion was first recorded 32 years ago (Rathor and Toqir 1980; Rathor *et al*. 1983) in Sindh Province. In the present study we noted that in district Mirpur Khas, *An. subpictus* remained resistant to DDT and Malathion, and there was no sign of reversal of resistance. Due to the development of resistance to Malathion, the use of Malathion was discontinued in 1996, and it was replaced by Deltamethrin, which is still being used.

A study on Pesticide susceptibility status of Anopheles mosquitoes in four flood-affected districts of South Sindh, Pakistan showed that *Anopheles subpictus* remained resistant to DDT and Malathion. In a study conducted by Rathor *et al.*, *Anopheles* mosquitoes were reported to show resistance against the three commonly used pyrethroids- permethrin, lambda-cyhalothrin and deltamethrin [12]. The results of a study showed first indication of pyrethroids resistance in *An. Subpictus*, with widespread, multiple resistances to organochlorine and some report of tolerance to organophosphate insecticides and recently to pyrethroids in a malarious area, from southern Iran [11]. However, with appropriate resistance-management strategies, the development of high levels of resistance can be prevented or delayed. The results of this study will provide a clue for monitoring and mapping of insecticide resistance in the malaria vector and an important evidence base for strategic planning for vector control.

Conclusion

This is the preliminary survey of insecticide resistance/susceptibility of anopheles species in District Mirpur Khas. It provide evidence base data on insecticide resistance, which enable to follow trends in susceptibility status in this area and will also serve as a base for resistance management interventions. In view of the present status of the resistance in disease vector, the development and implementation of comparatively new strategies for integrated vector management (IVM), needs to be planned in light of the data of this study. In order to manage, prevent, or slow the development of resistance to the presently used effective insecticides, a strategic approach for the judicious use of pesticides is essential. This approach requires efficient and regular monitoring of the susceptibility status of disease vectors as an important component of IVM. Unfortunately, pesticide resistance monitoring and surveillance is extremely inadequate in Pakistan. An effective resistance management policy is not possible without the strong evidence obtained from monitoring and surveillance. Further research is needed to confirm the role of anopheles species in malaria transmission and to monitor the trend of insecticide resistance in malaria vectors. Genetic/molecular studies required for the isolation of resistant genes in prevalent vectors is also recommended to confirm the findings of this survey.

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