Laboratory and Semi-Field Evaluations on Lethal and Residual Effects of Temephos and Pyriproxyfen Insecticides to Control Malaria Mosquito Larvae, *Anopheles Stephensi* Liston

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Abstract

Background: The application of insecticides against vector mosquito larvae is a crucial step to control human malaria. Insecticide resistance is a major impediment to vector control strategies. The main aim of this study was to conduct laboratory and semi-field evaluations on lethal and residual effects of temephos and pyriproxyfen insecticides against malaria mosquito larvae, *Anopheles stephensi*.

Methods: Both susceptibility test and residual bioassay were performed to assess the lethal concentrations of each insecticide on 50% (LC_{50}) of the IV instars larval populations and their activity periods according to standard protocols of WHO. Nine and eleven different concentrations with two sets of control in each case were applied for temephos and pyriproxyfen, respectively. Data were analyzed using probit analysis and SPSS software.

Results: The LC_{50} and LC_{90} for temephos and pyriproxyfen under laboratory conditions were 0.4 and 0.63, and 1.69×10^{-4} and 4.036×10^{-4} ppm, respectively. Although the field strain of *An. stephensi* larvae was completely susceptible to pyriproxyfen, there was noticeable resistance (8% mortality at the diagnostic dose) to temephos in Nikshahr County, Southeast Iran. This is the first report of resistance to temephos for this malaria main vector in Iran. Depending on the applied variable doses, the residual effects of temephos and pyriproxyfen under semi-field conditions lasted maximally for 3 and 10 weeks, respectively.

Conclusion: The high lethal and residual effects of pyriproxyfen on mosquito larvae confer an unprecedented opportunity in vector control operations leading to elimination of malaria in Iran.

Introduction

Human malaria frequently contracted by the infectious bites of adult female *Anopheles* mosquitoes is one of the most important vector-borne infections in Iran though it is on the verge of elimination in endemic oriental parts of this country.[1] It is caused by the protozoan parasitic species in the genus *Plasmodium* which induces febrile paroxysms every two to three days marked with fever, chills, and sweats.[2] Although the majority of malaria

cases are due to *Plasmodium vivax*, both this species and *Plasmodium falciparum* occur sympatrically in Iran.[3, 4] Malaria transmission is heterogeneous, unstable and year-round.

The national key control intervention policies and strategies consists of four major measures: indoor residual spraying, free distribution and delivery of insecticide-treated nets to all families, foci (including larviciding) and case investigation, and free malaria diagnosis in the public sector and radical treatment of *P. vivax* cases by active and passive case detection.[5]

Although a total of five malaria vectors including *Anopheles stephensi*, *An. culicifacies*, *An. fluviatilis*, *An. superpictus*, *An. dthali* and a suspected one *An. pulcherrimus* have regionally been incriminated, only the first species is presently considered since it is a predominantly endophilic and endophagic vector of malaria in most of the Eastern Mediterranean region and the Indian subcontinent.[6] *An. stephensi* Liston (Diptera: Culicidae) is one of the main malaria vectors although *An. culicifacies* and *An. fluviatilis* also play important roles in malaria transmission in southeast Iran.

One of the preliminary control measures is to inhibit the vector breeding by using conventional groups of chemical compounds such as organochlorines, organophosphates, carbamates, and/or pyrethroids against mosquito pre-adult stages.[7, 8] Insecticide resistance is also increasingly prevalent. The extensive development of resistance in mosquitoes to insecticides thus poses a serious challenge to combat the transmission of infectious agents. One of the first vector control methods includes larviciding which has achieved major success in the past. As part of a routine national monitoring program and as a result of reduced insecticide effectiveness, nowadays certain guidelines are recommended by the World Health Organization (WHO) on the use of such compounds as temephos and pyriproxyfen.

These latter insecticides are the subject matter of the present article. The former (temephos), as an organophosphate (OP), has a very low toxicity for mammals and is a fast-acting insecticide. It has been used to control mosquito larvae such as *Anopheles* spp., *Culex* spp., and *Aedes aegypti*.[9] It has also been used in Africa and Central and North America to control black fly larvae.[10] The latter (pyriproxyfen) is an insect growth regulator (IGR) with slow release, delivery, and activity which mimics the juvenile hormone of larval insects.[11] It has been used against mosquitoes such as *An. stephensi*, *Culex pipiens*, *Culex tarsalis*, *Ae. aegypti*, and other medically-important insects.[12]

The extensive use of insecticides and the challenges of mosquito resistance to these chemical compounds are the main reasons to undertake this study. The main aim of the present investigation is to conduct laboratory and semi-field evaluations on the lethal effects of temephos and pyriproxyfen insecticides for the control of malaria mosquito larvae, *An. stephensi*. There appear that no previous reports exist on the lethal and residual effects of temephos and pyriproxyfen insecticides under laboratory and semi-field conditions to control malaria mosquito larvae, *An. stephensi*, in the endemic focus of Nikshahr, southeast Iran, where almost 12% of the total national malaria cases were observed in 2010.[13]

Materials and Methods

Study Area

The present study was carried out during a period of 12 months in Nikshahr county (26°12'N, 60°12'E) with an area of 23,930 km^2 at an altitude of 510 meters above the sea level on the south central part of Sistan-Baluchistan province, southeast Iran (Figure 1). It is the third largest county with five districts and a population of 148901. The mean annual ambient temperature and relative humidity of Nikshahr are 32 °C and 36.8%, respectively. This county is located in the Sistan-Baluchistan province bordering the neighboring country of Pakistan. It has a subtropical climate and is susceptible to seasonal transmission of malaria. The risk of malaria transmission in this endemic malaria area persists almost all the year round. The people are mainly involved in agricultural activities.

Mosquito Collection

Malaria mosquito larvae, *Anopheles stephensi*, were collected from rice paddies in the suburbs of Nikshahr using standard World Health Organization (WHO) dipper. The field collection of larval *An. stephensi* mosquitoes at Nikshahr was conducted during the malaria transmission season. Following their species identity confirmation, they were subjected to susceptibility tests or residual bioassays as below. Wild *An. stephensi* (*mysoriensis* strain) larvae were transferred from their natural habitats to the local insectarium. They were maintained at 22±2 °C at a relative humidity of 74±3%.

Insecticides

Two different technical grade insecticides were formulated as emulsifiable concentrate (EC) and slow delivery (SD) were used in the present study, respectively. They are known as:
- temephos (Temelod®) EC 50% at concentrations of 0.7 ppm, 0.525 ppm, 0.35 ppm, and 0.175 ppm.
- pyriproxyfen (Pyrilarv®) SD 0.5% at concentrations of 0.000624 ppm, 0.000312 ppm, 0.000156 ppm, 0.000075 ppm,
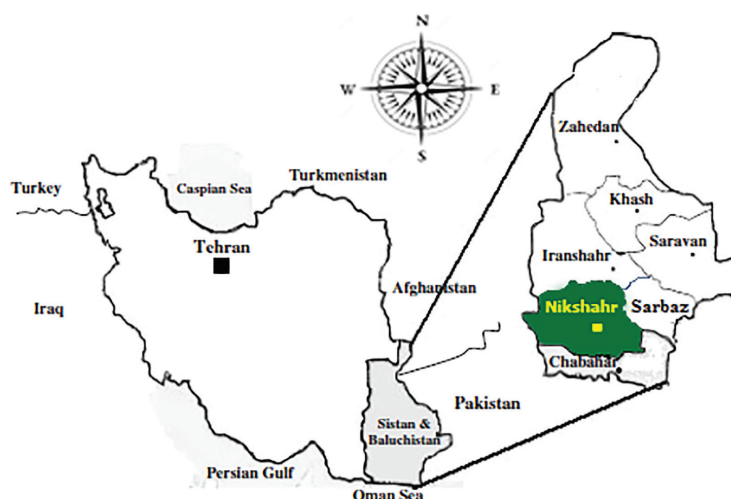
Figure 1: Location of the study area, Nikshahr County, Sistan-Baluchistan Province, an endemic malaria area in Iran

Both of them were produced by Levant Overseas Developments Ltd. (France) were examined in the following experiments.

Susceptibility Tests

Actively swimming early IV instars larvae were used in these experiments. Two different insecticides were used on field-collected larval mosquitoes of *An. stephensi* (*mysoriensis* strain) at Nikshahr. Insecticide susceptibility tests with temephos or pyriproxyfen against *An. stephensi* larvae using standard methods were conducted under laboratory conditions.[12] Nine and eleven different concentrations of temephos and pyriproxyfen larvicides were tested, respectively. At each concentration, a total of 100 larvae in four replicates of 25 larvae were tested. Two replicates of 25 larvae were used as control in each test. The larvae were fed with fish food, and mortality due to temephos exposure and adult emergence inhibition (AEI) due to the insect growth regulator (IGR) was scored after 24 hr of recovery period. Moribund larvae (presenting tremors, rigidity or mobility to reach water surface on touch) were considered as dead. Abbott's formula was used to correct the observed mortality of the larvae. All the data were corrected if the control mortality is between 5 and 20%.[14]

Semi-Field Residual Bioassays

In an area next to rice paddies, ten pits (8 tests, 2 controls) with dimensions of 1×1×0.5 meters were made in the ground. Each pit was insulated from all sides by double layered polyethylene plastic sheets. These pits were filled with river water to a depth of 40 cm. A total of 4000 laboratory-bred *An. stephensi* (*mysoriensis* strain) mosquito larvae were examined for temephos and pyriproxyfen. Batches of 100 actively late 3rd instar larvae were added into these pits weekly. Four different concentrations

(recommended by factory and WHO) for each of these insecticides were used. They were uniformly sprayed onto pit surfaces using 2 l capacity spraying atomizers. Following exposure, the larval mosquitoes were allowed to forage on fish food again. The pits were covered with fine mesh bed nets to prevent the escape of adult mosquitoes, to inhibit entry by free female ovipositing mosquitoes, and to keep debris away. The mortality of larvae to adults was recorded for temephos every 48 hours. The developments of larvae to adults were daily monitored and recorded 9-11 day later for pyriproxyfen. These continued until mortality dropped to below 75% and the times taken were recorded. The percentage of adult emergence inhibition (AEI %) following exposure to the IGR larvicide, pyriproxyfen, was calculated accordingly:

$$EI(\%) = 100 - \left(\frac{T \times 100}{C}\right)$$

Where T and C are the % of adult mosquito emergence from treated and control sites, respectively.

Data Analysis

Data were analyzed using Probit analysis[14] to determine the 50% lethal concentration values (LC_{50}) and 90% lethal concentration values (LC_{90}) of the field strain. Control mortality was corrected using Abbotts' formula. A statistical analysis of LC_{50} and LC_{90} was based on the overlap of 95% confidence intervals.

The data were analyzed statistically using SPSS version 19 software.

Results

Laboratory Susceptibility Tests

Temephos: From all different concentrations of the insecticide, the four lowest concentrations of

temephos that showed 5-95% mortality were chosen for probit analysis (Table 1). After analyzing these data and drawing the regression line, the LC_{50} and LC_{90} of temephos larvicide against *An. stephensi* larvae were 0.4 ppm and 0.63 ppm, respectively (Table 2, Figure 2). According to WHO criteria, a 98–100% mortality rate indicates susceptibility, 80-97% mortality rate indicates tolerance (requires confirmation of resistance with other methods), and <80% mortality suggests resistance. WHO diagnostic dose of temephos is 0.25 mg/l for *An. stephensi*. The data obtained in the present study indicated that there was noticeable resistance (8% mortality at diagnostic dose) of *An. stephensi* larvae to temephos larvicide in Nikshahr County, Iran. This is the first report of resistance to temephos for this main malaria vector in Iran.

Pyriproxyfen: In the case of the IGR insecticide, pyriproxyfen, the four lowest concentrations gave the AEI lower than full scale (%100) (Table 3). Therefore, the seven higher concentrations of pyriproxyfen exhibited full scale adult emergence inhibition. It is important to note that at the 0.00015 mg/l applied concentration of pyriproxyfen, 50% of *An. stephensi* adult mosquitoes were inhibited to emerge at laboratory conditions of 22±2 and relative humidity of 74±3%. After analysis of these data and drawing the regression line, the LC_{50} and LC_{90} of pyriproxyfen IGR larvicide against *An. stephensi* larvae were $1.69×10^{-4}$ ppm and $4.036×10^{-4}$ ppm, respectively (Table 4, Figure 3). The data showed that the field strain of *An. stephensi* larvae in this malaria area is completely susceptible to pyriproxyfen.

This experiment showed that the percentage of mortality of adults, larvae and pupae were 1.17, 8.12, and 90.7%, respectively. The mortality of pupae was, thus, the highest among all the tested mosquito stages.

Semi-Field Residual Bioassays

Temephos: The residual effectiveness of four different concentrations of temephos insecticide against *An. stephensi* (*mysoriensis* strain) mosquito
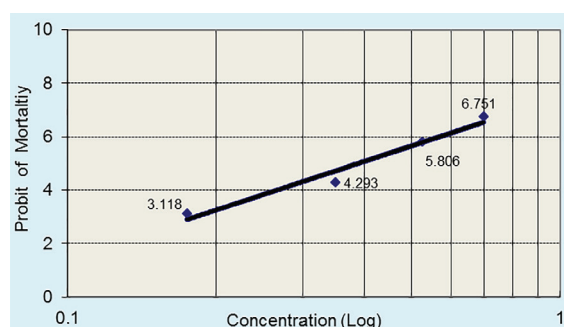

Figure 2: The regression line of *Anopheles stephensi* (*mysoriensis* strain) larval mortalities following exposure to four different concentrations of temephos under laboratory conditions

Table 1: Effects of different concentrations of temephos on *Anopheles stephensi* (*mysoriensis* strain) larvae in Nikshahr

ppm	Temephos concn. ml/l	Total no. larvae	No. viable larvae	No. dead larvae	% Mortality
22.4	0.0224	100	0	100	100
11.2	0.0112	100	0	100	100
5.6	0.0056	100	0	100	100
2.8	0.0028	100	0	100	100
1.4	0.0014	100	0	100	100
0.7	0.0007	100	0	96	96
0.525	0.000525	100	21	79	79
0.35	0.00035	100	76	24	24
0.175	0.000175	100	97	3	3
	Control 1	100	100	0	0
	Control 2	100	100	0	0

Table 2: The outcome of probit regression analysis due to the effect of four different concentrations of temephos insecticide on *Anopheles stephensi* (*mysoriensis* strain) larvae in Nikshahr

Concentration [ppm] (d)	Log dose (x)	Sample size (n)	Mortality (r)	Corrected Mortality (P)	Probit y'	Expected Probit (y)	95% fiducial limits	
							(Lower)	(Upper)
0.175	-0.75696	100	3	3	3.119	2.549	-0.164	5.263
0.35	-0.45593	100	24	24	4.294	4.555	3.448	5.662
0.525	-0.27984	100	79	79	5.806	5.728	4.742	6.715
0.7	-0.1549	100	96	96	6.751	6.561	5.048	8.073

The regression line is: y=7.59+6.66 x
The standard error of Slope is 0.59
The degree of freedom is 2
The Chi-square value is 11.95
The LC50 is 0.408
The lower limit of 95% confidence limits of LC50 is 0.054
The upper limit of 95% confidence limits of LC50 is 0.655
The LC90 is 0.635

Table 3: Effect of different concentrations of pyriproxyfen insecticide on *Anopheles stephensi* (*mysoriensis* strain) larvae

Pyriproxyfen concn. mg/l(ppm)	Total no. larvae	No. dead larvae	Total no. pupae	No. dead pupae	No. viable emerging adults	No. dead adults	% AEI
0.08	100	5	95	95	0	0	100
0.04	100	7	93	93	0	0	100
0.02	100	8	92	92	0	0	100
0.01	100	6	94	94	0	0	100
0.005	100	8	92	92	0	0	100
0.0025	100	7	93	93	0	0	100
0.00125	100	10	90	90	0	0	100
0.00062	100	8	92	89	3	1	97.90
0.00031	100	9	91	65	26	3	75.92
0.00015	100	6	94	39	55	7	49.73
0.000075	100	2	98	7	91	0	4.71
Total	1100	76	1024	849	175	11	---
Control 1	100	2	98	3	95	0	---
Control 2	100	3	97	3	94	0	---

Table 4: The outcome of probit regression analysis due to the effect of four different lethal concentrations of pyriproxyfen insecticide on *Anopheles stephensi* (*mysoriensis* strain) larvae in Nikshahr

Concentration [ppm] (d)	Log dose (x)	Sample size (n)	Mortality (r)	Corrected Mortality (P)	Probit y'	Expected Probit (y)	95% fiducial limits	
							(Lower)	(Upper)
0.000075	-4.12494	100	9	9	3.659	3.794	2.963	4.624
0.000156	-3.80688	100	52	52	5.05	4.876	4.377	5.376
0.000312	-3.50585	100	77	77	5.739	5.901	5.273	6.529
0.000624	-3.20482	100	98	98	7.054	6.925	5.883	7.968

The regression line is: y=17.83+3.40 x
The standard error of Slope is 0.28
The degree of freedom is 2
The Chi-square value is 4.1
The LC50 is $1.69×10^{-4}$
The lower limit of 95% fiducial limits of LC50 is $1.123×10^{-4}$
The upper limit of 95% fiducial limits of LC50 is $2.423×10^{-4}$
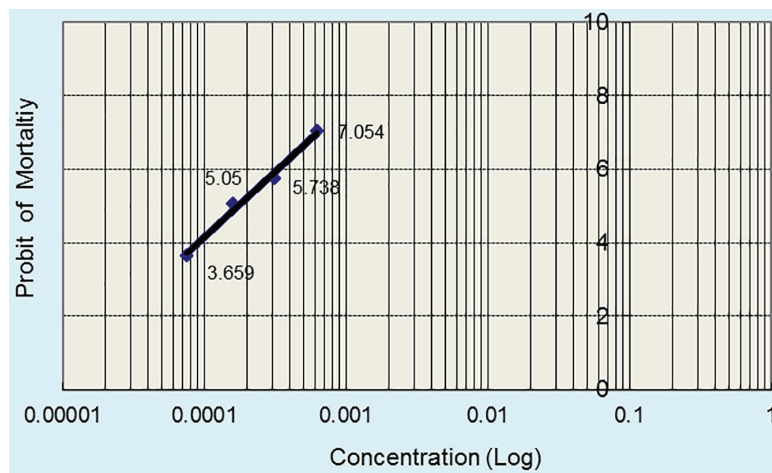The LC90 is $4.036×10^{-4}$

Figure 3: The regression line of *Anopheles stephensi* (*mysoriensis* strain) larval mortalities following exposure to four different concentrations of pyriproxyfen under laboratory conditions

larvae indicated that the two upper concentrations (0.015, 0.0224 ml/l) produced positive residual effects of temephos during the 28-day experimental period. It also showed that after the first week all doses revealed 100% mortality, but after 14 days only the lowest concentration (0.01 ml/l) demonstrated less than full scale mortality (77%). The maximal duration of the residual effect of temephos under semi-field conditions was three weeks (Figure 4). Furthermore, there was no significant difference between the mortality percentages at the three higher concentrations of insecticide after three weeks of exposure (P>0.05).

Pyriproxyfen: The residual effectiveness of four different concentrations of pyriproxyfen insecticide against *An. stephensi* (*mysoriensis* strain) mosquito larvae indicated that the two upper concentrations
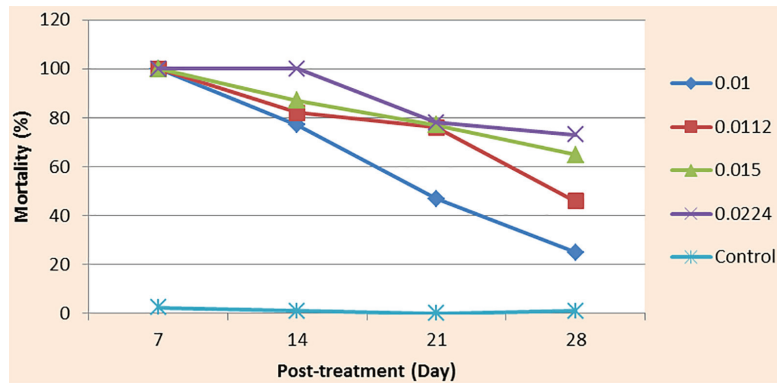
Figure 4: The residual outcome of four different concentrations of temephos insecticide (0.01, 0.0112, 0.015, 0.0224 ml/l) on *Anopheles stephensi* (*mysoriensis* strain) larvae in Nikshahr
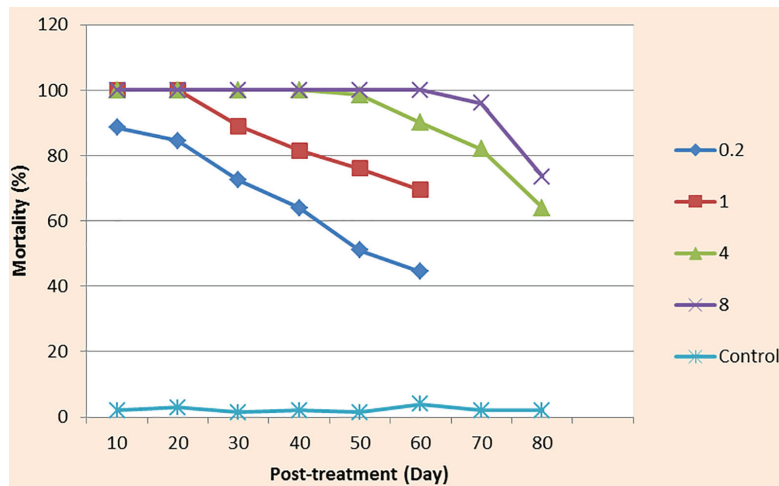

Figure 5: The residual outcome of four different concentrations of pyriproxyfen insecticide (0.2, 1, 4, 8g ai/ha) on *Anopheles stephensi* (*mysoriensis* strain) larvae in Nikshahr

(4, 8g ai/ha) produced positive residual effects during the first 50-day experimental period. The residual mortalities at these two concentrations were also positive. The maximal duration of the residual effect of pyriproxyfen under semi-field conditions was ten weeks (Figure 5).

Discussion

Temephos has been used for many years as an effective larvicide in malaria control program in southern Iran.[7] Many studies have been done in Iran and other countries on the susceptibility level of *An. stephensi* to different pesticides such as temephos.[16, 17] LC_{50} and LC_{90} values of temephos for *An. stephensi* were reported 0.0035, and 0.0131 mg/l respectively in north-western Rajasthan, India.[18] In a recent study, laboratory evaluation of temephos against *Anopheles stephensi* was done in Iran. In this research mosquitoes were collected from Kazerun, Fars province and for this strain LC_{50} and LC_{90} were calculated 0.0523, and 0.3822 mg/l, respectively.[19]

In another study conducted in Iran, susceptibility of some geographical strains of *An. stephensi* was studied to temephos. Eight different areas in two important endemic malaria provinces were considered

as field collecting sites (Hormozgan and Sistan-Baluchistan provinces). The results indicated that the LC_{50} ranged from 0.0022 mg/l to 0.0141 mg/l for *An. stephensi* to temephos. Almost all the strains were susceptible to temephos and low level of resistance ratio (RR) was noticed in all tested populations except for the Chabahar strain (RR=4.27 fold).[16]

Chabahar Sea Port is located near the Oman Sea where resistance of *An. stephensi* to Temephos was confirmed by Anderasen in Oman for the first time in the Middle East region.[20] Different levels of resistance to larvicides were reported in anopheline malaria vectors worldwide. *An. stephensi* larvae from India were reported to be resistant to a number of insecticides including temephos.[15] Low level of larval temephos resistance was reported for this main malaria vector in Pakistan.[21]

Despite earlier reports on the susceptibility of this main malaria vector to this insecticide in other endemic foci within the oriental region of Iran, the results of the present study for the first time in Iran confirmed the resistance of *An. stephensi* larvae to temephos insecticide at the diagnostic dose concentration. Therefore, it could be an important alarm in the development of resistance to temephos

in Iranian strain of *An. stephensi*.

In another study, the efficacy of a 0.5% pyriproxyfen granule (Surmilarv W 0.5G, Sumitomo Chemicals) was assessed for the control of *Anopheles gambiae s.s.* and *Anopheles arabiensis*. These species were highly susceptible to pyriproxyfen. Estimated emergence inhibition values were very low and similar for both species. The minimum dosages that completely inhibited adult emergence were between 0.01-0.03ppm. An application of 0.018 ppm prevented 85% of adult emergence over six weeks.[22]

The efficacy of pyriproxyfen was evaluated against the dengue vector *Aedes aegypti*. Larval bioassays were carried out on susceptible mosquito larvae, and for this species, LC_{50} and LC95 were 1.1×10^{-4} and 3.2×10^{-4} mg/l, respectively.[23] The results of this study were similar to our study.

The results of our study indicated that Pyriproxyfen acted at very low concentrations by inhibiting the adult emergence of *An. stephensi* (97.90 % inhibition rates at 6.2×10^{-4} mg/liter). According to the current results, *An. stephensi* larvae in this endemic malaria area are completely susceptible to pyriproxyfen.

Pyriproxyfen showed high toxicity against mosquito pupae but less action on larvae and adults. It is considered as an effective insecticide for malaria control because it shows good insecticidal activity against target pests and low risk to humans.

The present study demonstrated a three-weeks persistence of the relative efficacy of temephos *in situ*. The maximal residual time of insecticides was estimated to be about ten weeks for pyriproxyfen. There was no significant difference between the mortalities of *An. stephensi* on different sprayed surfaces (P>0.05). Furthermore, it was concluded in the present study that pyriproxyfen, which is a third generation insecticide, revealed a longer residual effect than temephos in endemic malaria areas.

In another study, the residual activities of pyriproxyfen and temephos were studied in Malaysia against *Aedes aegypti*. Pyriproxyfen possessed the longest residual activity in both indoor (43 weeks) and outdoor (26 weeks) conditions, followed by temephos (26 weeks in indoor and 16 weeks in outdoor).[24]

Another study was done in Brazil in order to evaluate the mortality and residual effects of some larvicides against *Aedes aegypti* under simulated field conditions. The results showed that temephos and pyriproxyfen caused 100% larval mortality in both populations (susceptible and temephos resistant) for 60 days after treatment.[25, 26]

The results of these studies are partially different from those of our study. These differences can be attributed to several factors such as different geographic conditions, behavioral differences between the species, and study conditions. Moreover, laboratory tests on adults of this malaria vector have contributed to its behavior.[27]

Conclusion

It is concluded from the results that although the tested strain was completely resistant to temephos, the IGR insecticide, pyriproxyfen, is still an effective means of control against *Anopheles stephensi* (*mysoriensis* strain) larvae in this endemic malaria area in Iran.

Acknowledgement

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