Resistance status of main malaria vector, *Anopheles stephensi* Liston (Diptera: Culicidae) to insecticides in a malaria Endemic Area, Southern Iran

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ARTICLE INFO

Objective: To evaluate the susceptibility of *Anopheles stephensi* (An. stephensi) Liston, the main malaria vector in southern Iran, to WHO recommended insecticides. Methods: Larvae of *An. stephensi* were collected from three different larval habitats in both urban and rural area of Bandar Abbas city and one rural area in Rudan county southern Iran. WHO standard method was used for evaluation of adult and larval mosquito susceptibility. Bendiocarb, permethrin, lambda-cyhalothrin, deltamethrin as insecticide and temephos and chlorpyriphos as larvicide were used at the diagnostic dosages recommended by WHO. Results: Findings of this study showed all larval populations of *An. stephensi* were completely susceptible to temephos and candidate for resistance to chlorpyriphos. Adult mosquitoes in rural areas of Bandar Abbas city were resistant to pyrethroid and carbamate insecticides. Conclusion: Comparison of the results of this survey with previous studies indicates that the resistance to pyrethroids and carbamates in this malaria endemic region is increasing. Wide use of pesticides in agriculture is certainly effective in increasing resistance. The inter-sectoral coordination and collaboration in health and agriculture seem to be necessary to manage insecticide resistance in malaria vectors.

1. Introduction

Malaria is considered one of the most important infectious blood diseases in tropical and subtropical developing countries around the world. In 2016, an estimated 216 million cases of malaria occurred worldwide, of which 445 000 has resulted in deaths[1]. Malaria elimination is the common goal of World Health Organization (WHO) and the health system in Iran. Elimination of malaria is defined as the reduction to zero of the incidence of locally acquired infection from human malaria parasites in a defined geographical area as a result of planned attempts[2]. Following a decline in malaria cases in recent years, the malaria elimination program, technically supported by WHO, has initiated since 2009 in Iran[3].
Due to successful implementation of malaria control plan, a reduction trend from 1,847 to 81 cases between 2010 and 2016 was found in indigenous malaria positive cases in Iran[1].

Long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS) and larviciding are the most powerful and most broadly applied interventions against malaria vectors. These methods work by reducing human-vector contact and decreasing the lifespan of female mosquitoes so that they do not survive long enough to transmit the parasite[4,5]. Other personal protection method is to wear impregnated clothes for military and other field workers in the malaria endemic areas[6]. In the short time after the 1940s, when insecticides were widely used for the first time in the world, insecticide resistance, which has unintended side effects in controlling malaria vectors, has been raised. Identifying and preventing the development and spread of insecticide resistance in malaria endemic countries are very important and the program for monitoring and managing insecticide resistance is one of the national strategies of these countries. The emergence and expansion of resistance over the last two decades has referred to a high dependence on only one group of insecticides (pyrethroids) for both public health and agricultural purposes.

In order to maintain significant gains in malaria control and the success of malaria elimination program, it is critical to prevent further development of resistance and to maintain the effectiveness of vector control interventions[7]. Since 2010, resistance to at least one class of insecticides has been reported in at least one malaria vector species in 60 of the 96 malaria-endemic countries that conducted monitoring; also, 49 countries reported resistance to at least two classes of insecticides[7].

There are seven Anopheles mosquitoes as the malaria vectors in Iran, i.e. Anopheles stephensi (An. stephensi), Anopheles culicifacies s.l., Anopheles superpictus s.l., Anopheles fluviatilis s.l., Anopheles maculipennis complex including Anopheles sacharovi and Anopheles dthali[8,9]. Despite the implementation of the malaria control program over the years, malaria is still a major health problem in the southern regions of Iran. Most local malaria transmission in Iran occurs in Sistan va Baluchestan, Hormozgan and Kerman provinces[2,10].

There is a long history of using insecticides and resistance to them in malaria vectors of Iran. Earlier studies showed resistance of An. stephensi to DDT[11], dieldrin[12], malathion[13], lambda-cyhalothrin[14] and deltamethrin[15,16]; Anopheles culicifacies s.l. to DDT[17], Anopheles dthali to DDT[18], and Anopheles maculipennis complex to DDT, propoxur, bendiocarb and malathion[19] in Iran. In recent years, pyrethroids have been used for residual spraying and long-lasting bed net treatment in national malaria control program.

The distribution of An. stephensi is from China, Thailand, Bangladesh, India, Pakistan, Afghanistan, Iran, Iraq, Oman, Myanmar (Burma), Nepal, Bahrain, Saudi Arabia and Egypt[8]. According to the earlier studies, An. stephensi is the dominant anopheine species in coastal areas of southern Iran[8]. This species is the main and the most important malaria vector in Hormozgan province, especially in plane areas[20].

One of widely used methods for identifying resistance to insecticides in mosquitoes is a simple response-to-exposure test developed by world health organization[21]. This test will differentiate between susceptible and resistant populations of mosquitoes and intended to be used as a field and laboratory surveillance tool[21]. The aim of this study was to evaluate the current susceptibility of An. stephensi to common chemical insecticides in Southern Iran, for implementing in vector control program.

2. Materials and methods

2.1. Study area

This survey was conducted on the samples of An. stephensi collected from Bandar Abbas and Rudan counties, Hormozgan province, Southern Iran.

Bandar Abbas (with an average altitude of 9 m above sea level and geographical coordinates: 27°11'11.4”N; 56°16'50.9”E), the capital city of Hormozgan province, is located on the southern coast of Iran, north of the Persian Gulf (Figure 1). This city has strategic location in the Strait of Hormuz and is the main site of the naval base of Iran. According to the Koppen BWh climate classification, the area has hot desert climate (tropical), maximum temperature at summer can reach 49 °C in summer and the minimum temperature may drop to 5 °C in winter. Annual precipitation is about 170 mm and average relative humidity is 65%.

Rudan County is composed of topographic and mountainous features. The plain and smooth sections cover most of the central and southern regions. The lands of this area are often flat and between 150 and 700 meters above sea level. The mountainous area has significant heights in the north, west and east. It has a very hot and dry climate, so the average of annual temperature shows the highest degree and lowest relative humidity percentage in Hormozgan province. The average annual precipitation in this county is 250 mm.
2.2. Sampling

This study was carried out from February until the end of May 2018 with aims of finding the best larval habitats for An. stephensi, sampling and conducting the susceptibility tests. Geographic coordinates of the larvae and adult mosquito collection sites were recorded by using a GPS device. Larval stage of An. stephensi was collected from different larval habitats in the urban area of Bandar Abbas city (27°12′18.71″N; 56°17′32.11″E), Hormoodar village (27°19′14.72″N; 56°19′14.80″E) in Bandar Abbas County, and Deh Matoon village (27°30′43.05″N; 57°17′50.61″E) in Rudan County, by using standard dipping method. Physical characteristics of the breeding sites were registered in the relevant forms and chemicals elements were tested in water quality laboratory, Hormozgan University of Medical Sciences. The collected larvae were transferred to the insectary for rearing to adults. Hormoodar population successfully reared and eggs float ridges for detection of An. stephensi biotype was studied[22]. A number of specimens from each breeding site were mounted and identified using morphological key[23]. Breeding sites with An. stephensi were considered for further sampling and tests.

2.3. Adult susceptibility tests

According to the guideline developed by WHO[21], diagnostic dose of insecticides was used against female adult and larvae of An. stephensi mosquitoes. A total of four replicates were used as exposure (20-25 mosquitoes per test, totally 100 specimens) for each insecticide and two replicates as control (totally 50 specimens). Test kits and insecticide-impregnated papers were purchased from the WHO collaborative center in University Sains Malaysia (USM), Penang, Malaysia. Bendiocarb 0.1% (Batch No.159, Expiry date: March 2019), permethrin 0.75% (Batch No. 428, Expiry date: August 2018), lambda-cyhalothrin 0.05% (Batch No. 262, Expiry date: July 2018) and deltamethrin 0.05% (Batch No. 527, Expiry date: August 2018) were used against adult mosquitoes. The control test mosquitoes were exposed to papers impregnated only with the appropriate carrier oil for pyrethroids (Batch No. 262, Expiry date: August 2018) and carbamates (Batch No. 181, Expiry date: July 2020); that was without insecticide.

Tests were performed on the F1 progeny of wild-caught adult females of Hormoodar population, 3–5 days old fed with sugar. The mosquitoes were exposed to different insecticides by 60-minutes exposure time and 24 hours recovery period. A pad of a cotton wool soaked in 10% sugar solution was provided as feeding source of mosquitoes during the recovery period. Tests were carried out in an insectary maintained at 27±2°C temperature and (75±10)% relative humidity, 14:10 light: dark[21]. Mortality rate for each test was estimated at the end of tests.

2.4. Larval susceptibility tests

Two larvicides, i.e. temephos (0.25 mg/L) and chlorpyriphos (0.025 mg/L) were used at the diagnostic dose provided by WHO24]. Collected larvae from breeding places were transferred to insectary of Bandar Abbas Research Station. After 24 h recovery time, late 3rd or early 4th instars were used for the tests by WHO test procedure for mosquito larvae[25] and mortality rate was recorded after 24 h exposure time. Totally 100 specimens were used per each test and 50 specimens per each control. Tests were carried out in an insectary maintained at (27±2)°C temperature and (75±10)% relative humidity.

2.5. Data analysis

If the results of control mortality were less than 5%, the tests were considered to be correct, but in the cases of between 5%–20%, it is necessary to correct by Abbots’ formula and for more than 20% the tests were discarded and repeated by new specimens. Based on the latest guideline of WHO, mortality of the test between 98%–100% was considered as susceptible, 90%–97% as candidate of resistance that should be confirmed using specific methods, and less than 90% was considered as resistant[21].

3. Results

The physical condition and chemical elements of the larval habitats of An. stephensi during the study period is shown in Tables 1 and 2. The biotype of An. stephensi that was reared from Hormoodar was intermediate because the number of eggs ridges was 15.44±0.82.

<table>
<thead>
<tr>
<th>Larval habitat characters</th>
<th>Hormoodar natural habitat</th>
<th>Hormoodar artificial habitat</th>
<th>Urban habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat type</td>
<td>River</td>
<td>Cement pool</td>
<td>Leakage</td>
</tr>
<tr>
<td>Substrate</td>
<td>Sandy</td>
<td>Cement</td>
<td>Muddy</td>
</tr>
<tr>
<td>Sunlight</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Algae</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vegetation</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Permanency</td>
<td>Permanent</td>
<td>Temporary</td>
<td>Temporary</td>
</tr>
</tbody>
</table>

As shown in Table 3, our findings showed resistance to both pyrethroid and carbamate insecticides with mortality rates of
(84.31±2.62)%, (89.66±2.34)%, (82.27±5.20)% and (86.10±3.61)% to lambda-cyhalothrin, deltamethrin, permethrin and bendiocarb, respectively. Indications of resistance to chlorpyriphos were observed with mortality rates of (96.22±1.40)%, (96.67±0.70)% and (97.92±0.99)% in Deh Matoon, Bandar Abbas city, and Hormoodar populations. Findings of this study showed three populations (Hormoodar, Deh Matoon, and Bandar Abbas city) of An. stephensi from Bandar Abbas and Rudan Counties, Southern Iran, were completely susceptible to temephos and candidate of resistance to chlorpyriphos in the diagnostic dose recommended by WHO. An. stephensi showed resistance to both pyrethroid and carbamate insecticide groups that used in this study (lambda-cyhalothrin, deltamethrin, permethrin, and bendiocarb) in Hormoodar population.

### Table 3

Response of Anopheles stephensi collected from Southern Iran to different insecticides/larvicides, 2018.

<table>
<thead>
<tr>
<th>Population</th>
<th>Insecticide/Larvicide</th>
<th>Exposure time (h)</th>
<th>Total Mortality (%) Mean±SD</th>
<th>Resistant status</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hormoodar</td>
<td>Bendiocarb 0.1%</td>
<td>1</td>
<td>223</td>
<td>86.10±3.61</td>
</tr>
<tr>
<td></td>
<td>Permethrin 0.75%</td>
<td>1</td>
<td>220</td>
<td>82.27±5.20</td>
</tr>
<tr>
<td></td>
<td>Lambda-cyhalothrin 0.05%</td>
<td>1</td>
<td>102</td>
<td>84.31±2.62</td>
</tr>
<tr>
<td></td>
<td>Deltamethrin 0.05%</td>
<td>1</td>
<td>116</td>
<td>89.66±2.34</td>
</tr>
<tr>
<td></td>
<td>Temephos (0.25 mg/L)</td>
<td>24</td>
<td>100</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td></td>
<td>Chlordpyriphos (0.025 mg/L)</td>
<td>24</td>
<td>101</td>
<td>97.92±0.99</td>
</tr>
<tr>
<td>Bandar Abbas City</td>
<td>Temephos (0.25 mg/L)</td>
<td>24</td>
<td>100</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td></td>
<td>Chlordpyriphos (0.025 mg/L)</td>
<td>24</td>
<td>99</td>
<td>96.67±0.70</td>
</tr>
<tr>
<td>Deh Matoon</td>
<td>Temephos (0.25 mg/L)</td>
<td>24</td>
<td>106</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td></td>
<td>Chlordpyriphos (0.025 mg/L)</td>
<td>24</td>
<td>106</td>
<td>96.22±1.40</td>
</tr>
</tbody>
</table>

R= Resistance, S= Susceptible, CR= Candidate of resistance.

First clues of pyrethroid resistance was found at 2011 from Iran[15] and then confirmed in another study conducted in Hormozgan province[27]. The results of a new study at 2015 conducted in Chabahar district, Sistan va Baluchestan province, showed that An. stephensi was resistant to DDT, tolerant to malathion, propoxur, cyfluthrin, and lambda-cyhalothrin and susceptible to deltamethrin[18]. Another study in this district determined tolerance to deltamethrin and permethrin and resistance to lambda-cyhalothrin[16]. Two earlier studies in Jask district, east of our study area in Hormozgan province, showed An. stephensi was susceptible to pyrethroids that we used in our study, although it was resistant to DDT and dieldrin[14,31]. A recent study in Hormoodar area showed An. stephensi was candidate of resistance to deltamethrin with 91% mortality rate[32]. In the Punjab province, Pakistan, An. stephensi was susceptible to permethrin and deltamethrin, while confirmed or potential resistance to cypermethrin, λ-cyhalothrin, and cyfluthrin was observed from all the study sites[33]. According to a report published in 2016, An. stephensi was resistant against all tested insecticides in Afghanistan[34].

Regarding carbamates, which are using in rotation with pyrethroids in the national malaria vector control program in Iran, first indication of potential resistance in An. stephensi was reported from the southeast of Iran, with 95% mortality[27]. Although next study conducted in the area[35] resulted to 99.3% mortality, a study conducted in 2015 in 10 sentinel sites in southern Iran (Ministry of Health, Unpublished data) confirmed resistance to bendiocarb (86.41% mortality) in Kerman province and potential resistance to this insecticide in Sistan va Baluchestan province. In a cross-sectional study at 2010, An. stephensi (Minab population) was susceptible to bendiocarb and deltamethrin (100% mortality rate);

### 4. Discussion

An. stephensi is the main vector responsible for transmission of the pathogen that causes human malaria, i.e. *Plasmodium vivax* and *Plasmodium falciparum*, in the northern plates of Persian Gulf[24]. It considered being endophagous and endophilic, so it is exposed to the insecticides used in malaria vector control program. With the prolonged and continuous use of insecticides, the selection of insecticide resistant individuals occurs, increasing populations of mosquitoes more tolerant and less sensitive to the product and, therefore, promoting the reduction of the effectiveness of insecticides[20].

Among the insecticide used in this study, only temephos induced 100% mortality. Earlier studies showed An. stephensi was susceptible to this larvicide in south of Iran[26,27] and resistant in southwest[28]. A recent study showed that the altered enzymes lead to temephos resistance in An. stephensi[29]. There is a report of resistance to temephos from An. stephensi in the Al-Dhahira region from Oman country that located in the Middle East[30].
has increased in the past decade.

Selection of resistant individuals to pyrethroids was observed in major malaria vectors of the world, and the resistance genes are spreading rapidly throughout the world[37]. So, monitoring and mapping of insecticide resistance is an essential tool for implementation of malaria elimination in the areas under coverage of program. Although enzymes have role in the resistant of An. stephensi to DDT and pyrethroids[38] in Iran, it is found that piperonyl butoxide can suppress resistance to pyrethroid[32]. Therefore, it seems using synergists can be suggested as a new tool for prevention of pyrethroids resistance. However, there are a number of control strategies for combating against resistance such as rotation, mixture, using biological control and integrated vector management. These methods should be considered in vector control programs in future.

It can be concluded that resistance to pyrethroids in the main malaria vector of southern Iran is increasing. Extensive use of pyrethroids in agriculture is certainly effective in creating this event. The intersectoral coordination and collaboration in health and agriculture seem to be necessary to manage the resistance. Larviciding by temephos and Bacillus thuringiensis may be considered as the main method for control of An. stephensi in urban areas of Bandar Abbas, while it can help reduce the mosquito density in rural areas.

Conflicting interest statement

Authors declare that they have no conflict of interest.

Acknowledgements

The authors are very grateful to the staff of Bandar Abbas Research Station and Health Center of Hormozgan province for their kind collaboration during this study.


