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Laboratory and semi-field evaluation of *Bacillus thuringiensis* (Bioflash®) against *Anopheles stephensi* (Diptera: Culicidae) in an endemic malarious area of Iran

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Abstract

Background: *Anopheles stephensi* is considered to be the main malaria vector in the Middle East area including Iran. We aimed to evaluate the efficiency of a granule 10% formulation of *Bacillus thuringiensis* against this species under laboratory and semi-field conditions in an endemic malarious area of Iran.

Method: After collecting mosquitos from Hurmudar, in order to find the best effective dose, five dosages (a quarter-dose, half dose, recommended dose, a twice-dose and four times dose) were used for laboratory and semi-field assays in Bandar Abbas.

Results: Recommended dose by factory (0.017 g /0.1 m2) showed the highest mortality rate on *An. stephensi* larvae in both assays. The efficiency of Bt was very low (21 %) under semi-field condition. According to the results of this study, the use of bacteria alone cannot be a useful and effective way to control the vector of malaria in Iran's geographic conditions.

Conclusion: This method can be used (in the case of appropriate efficacy of the tested formulation in field condition) as one of the constituent parts of Integrated Vector Management (IVM) program along with other recommended methods.

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Introduction

Malaria is a parasitic disease transmitted by mosquitoes. According to the WHO annual report, the disease causes high mortality rates that are most commonly seen in children under 5 years old and impose a great deal of economic and health burden on human societies (1). This disease occurs all around the world more often in tropical and sub-tropical regions. Malaria is endemic in southern and southeastern parts of Iran and causes many health problems as one of the most important parasitic diseases in this region (2,3).

An. stephensi is a sub-tropical species and also an important vector of human malaria throughout the Middle East and South Asian region, including the Indo-Pakistan subcontinent, with a westward extension through Iran and Iraq into the Middle East

and Arabian Peninsula. This species is considered to be the main malaria vector in the Persian Gulf area (4,5). Previous studies have shown that *An. stephensi* is the most prevalent anopheline species in the malarious areas of southern Iran (4).

Due to the implementation of malaria control programs, the incidence of cases has decreased from 1847 to 57 during 2010 to 2017 (6). In Iran, based on the WHO recommended strategies for malaria vector control some methods including larviciding, widespread use of Long-lasting insecticide-treated bed nets (LLINs) and Indoor Residual Spraying (IRS) are running in malarious areas (2,3).

The control of mosquitoes in larval stages can act as an appropriate strategy to reduce the incidence of malaria cases. Different methods are used to control the immature stages of mosquitoes including the use of chemical insecticides, physical insecticides, surface films, insect growth regulators (IGR), larvivorous fishes, bacteria, fungi, nematodes and some viruses (2,7).

Today, scientists are focusing on the use of non-chemical alternative methods for mosquitoes control due to problems caused by the use of chemical insecticides which have environmental consequences and cause the development of physiological and behavioral resistance in mosquitoes (2,8).

In Iran, some native and non-native larvivorous fishes (*Gambusia spp* and *Aphanius spp*) and bacteria (*Bacillus thuringiensis* serotype H-14) were used as biological control of immature stages of malaria vectors (9).

Due to some ecological restrictions on the use of larvivorous fishes in breeding places especially drinking water sources, biological control in Iran has focused on the widespread use of bacteria as one of the components of Integrated vector management (IVM) program for malaria control. *Bacillus thuringiensis* is a gram-positive and aerobic bacterium that produces spores. The bacterium forms spores in the stationary phase of its growth cycle, and the dead spores produce a crystalline protein. After solubilization and proteolytic cleavage, it causes the death of larvae through the interaction of these toxins with the cells of the midgut epithelium (7,10).

Susceptibility level of vectors to all applied insecticides should be routinely evaluated each year in order to monitor the effectiveness of the used strategies. So, in this study we aimed to evaluate the efficiency of *Bacillus thuringiensis* against one of the most important malaria vectors (*Anopheles stephensi*) under laboratory and semi-field conditions in an endemic malarious area of Iran.

Materials and methods

Study area

This study was conducted during October to December 2017 in two laboratory and semi-field phases in Bandar Abbas Port and Hurmudar village in Hormozgan province (27° 11'N, 56° 16'E). This region is one of the endemic foci of malaria in Iran (Figure 1).

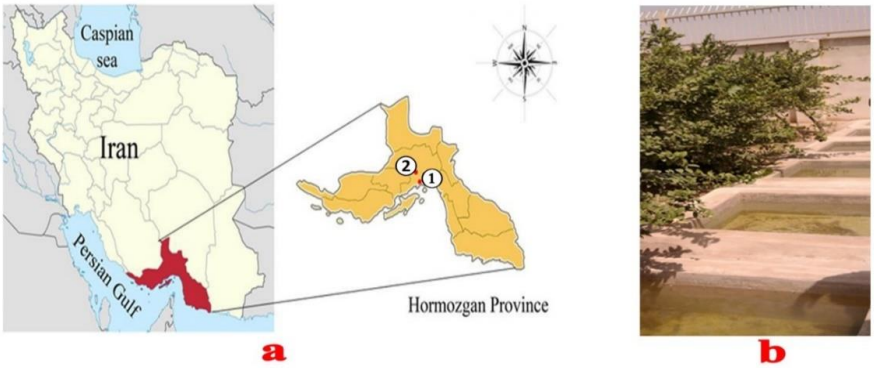

Figure 1. a: Maps of the sampling and study areas in an endemic malarious area, Hormozgan province, Iran. ①Bandar Abbas Port, ②Hurmudar Village

b: Artificial ponds for semi-field trial

Mosquito strain

In both laboratory and semi-field phases, the wild strain of *Anopheles stephensi* which was collected from Hurmudar village was used for all tests. Then, they were transferred to the Insectarium of the Department of Medical Entomology and Vector control, Health School of Hormozgan University of Medical Sciences. Larvae were kept at 25±3 ° C and 70-80 % relative humidity until the start of the tests.

Larvicide

In all tests, we used slow release formulation (granule 10%) of *Bacillus thuringiensis* M-H-14 (Bioflash®) with larviciding effect produced by Nature Bio Technology Company (Iran).

Laboratory assays

The collected samples were transferred to the laboratory in order to identify them based on their morphological characters by valid taxonomic keys. After the establishment of the field strains in the laboratory, the first generation of mosquitoes was used for all tests. This phase was carried out in the Insectarium of the department of medical entomology and vector control at Health School of Hormozgan University of Medical Sciences. Larvae were kept under the standard condition in order to reduce the confounding factors in the study.

The surface area of all used trays for test was 0.1 m^2. According to the larvicide guidelines, 170 g was recommended by the manufacturer for application to 1000 m^2. Based on this dose, we calculated the suitable amount for doing the tests for each tray (0.017 g). In order to find the best effective dose in laboratory condition on *An. stephensi*, five dosages (a quarter recommended dose, half recommended dose, recommended dose, twice recommended dose and four times recommended dose) were used for this phase of the study.

In both phases, 4 replicates were considered for each dose. Also, 2 control trays were considered for each test. The tests of treated groups were rejected if the control mortality was higher than 20% or when pupation was 10% of tested larvae. For each dose, 50 larvae (3^{rd} and 4^{th} instars) were released into each tray and the mortality rate was calculated after 24 hours.

Semi-field trial

For the implementation of semi-field trial, artificial ponds with dimensions of 1m × 1m × 0.5m were constructed in Bandar-Abbas and filled with drinking water. The ponds were covered with net (156 mesh per in^2) to prevent the breeding of wild mosquitoes. Based on the recommended dose for quarter, half, standard, twice and four times, we used 0.042, 0.085, 0.17, 0.34 and 0.68 grams, respectively (Figure 1). Water and air temperatures besides relative humidity were recorded for each test.

Results

For all treated groups with different dose of *Bacillus thuringiensis* under the laboratory condition, the recommended dose by factory (0.017 g /0.1 m2) showed the highest mortality rate on *An. stephensi* larvae. The results of all tested doses are shown in Table 1. In cases that the mortality rates in control group were 5-20%, they were corrected by Abbot's formula.

In semi-field trial, like the results of laboratory assay, recommended concentration had the highest mortality rates on this species, but with a much lower impact than laboratory tests (Table 2).

The average of water/air temperatures was recorded at 23.6/27.4◦C and 21.6/24.2 ◦C for laboratory and semi-field trials, respectively. The average relative humidity was 72.6% and 60.6% in laboratory and semi-field conditions, respectively.

Table 1. Mortality rate of *An. stephensi* with different doses of *Bacillus thuringiensis* after 24 hours under laboratory condition

		Dosages				
		Quarter (0.0042 g /0.1 m^2)	Half (0.0085 g /0.1 m^2)	Recommended (0.017 g /0.1 m2)	Twice (0.034 g /0.1 m^2)	Four times (0.068 g /0.1 m^2)
Mortality rate %	Treated groups	34*	81	94*	91	80*
	Control groups	7	3	6	3	7
Water temperatures ◦C		23	25	22	25	23
Air temperatures ◦C		27	28	27	28	27
Relative humidity %		72	75	71	75	70

*** Corrected with Abbot's formula**

Table 2. Mortality rate of *An. stephensi* with different doses of *Bacillus thuringiensis* after 24 hours under semi-field condition

		Dosages				
		Quarter (0.0042 g /0.1 m^2)	Half (0.0085 g /0.1 m^2)	Recommended (0.017 g /0.1 m2)	Twice (0.034 g /0.1 m^2)	Four times (0.068 g /0.1 m^2)
Mortality rate %	Treated groups	5	9	21	17	19
	Control groups	4	2	2	4	3
Water temperatures ◦C		22	21	21	23	21
Air temperatures ◦C		24	25	24	25	23
Relative humidity %		61	67	55	60	60

Discussion

Malaria is still a major parasitic disease in many countries, including Iran. larviciding is one of the most effective and suitable methods for controlling malaria vectors, especially in areas such as southern Iran where larval habitats are restricted (2,3). The idea of using *Bacillus thuringiensis* as an effective biological control has been used for many years in order to control some important mosquito-borne diseases (7,8,10-18).

This method is one of the highly recommended methods for controlling vectors due to its potential for using in drinking water, its specificity for some invertebrates, and relatively acceptable sustainability (8).

The effectiveness of this biologic agent has been studied in several countries on different medically important species (12-18).

In western Kenya, the efficacy of new water-dispersible granular (WDG) formulations of *Bacillus thuringienis* var.

israelensis (Bti; VectoBac) and *B. sphaericus* (Bs; VectoLex), (Valent BioScience Corp., Illinois, USA) for the control of larval *Anopheles gambiae sensu lato* Giles were evaluated under laboratory and field condition. The results showed that for 0.021 mg/l and 0.21 mg/l concentrations, the mortality rate was 50% and 95% after 24 hours, respectively (12).

In another study, a combination of *Bacillus thuringiensis var. israelensis* (Bti) and Arosurf MSF (Monomolecular Surface Film) were done against *Anopheles albimanus* in laboratory condition. More than 90% of post-exposure mortality was observed in this research (18).

Begum et al. in Australia examined the efficacy of Bti (VectoBac WG) on 3rd and 4th larval instars of *Aedes aegypti.* The highest and lowest mortality rates of 96.66% and 21.66% were obtained for doses of 1.0 µl/ml and 0.001 µl/ml, respectively (17).

Recently, the efficacy and duration of the effectiveness of a biolarvicide, Bactivec® SC (*Bacillus thuringiensis var. israelensis* SH-14) were evaluated in Bengaluru, India. According to their results, the tested formulation was effective and easy to handle. For the control of *Anopheles* and *Aedes* mosquitoes in freshwater habitats, 1 ml/50 l dosage was found effective in this research. Their results were not similar to our study because the formulation and geographical condition were completely different in these studies (10).

In 2012 a similar study was conducted in Iran, in this study laboratory and field evaluations of two formulations of *Bacillus thuringiensis* M-H-14 against mosquito larvae were done. Laboratory results showed a 50% reduction in larval density up to 7 days post-treatment when exposed to Bioflash® granule formulation. In semi-field trials, the percentage reductions of *An. stephensi* larvae were 28.2%–60.5% and 20.7%–44.9% at 3 dosages (2, 4 and 8 g/m2) of both formulations and, surprisingly, the highest dosage (8 g/m2) of granule formulation showed a lower reduction in larval density than the lowest dosage (2 g/m2) (7). Their results were completely in accordance with ours that by increasing the dose, the efficiency did not increase. They mentioned that Bioflash® had low larvicidal efficacy on mosquitos.

Any type of vector control program should be monitored and evaluated routinely and annually in order to maintain the efficiency of applied methods. Regarding the inclusion of Iran in the malaria elimination program and the importance of evaluating the used methods, we decided to evaluate one of the most important components of malaria vector control strategy that is highly recommended by Iranian Health System and also WHO. This study is the latest study in this field.

The present study evaluated the efficacy of *Bacillus thuringiensis* (Bioflash®) against immature mosquitoes under semi-field and laboratory conditions. Results showed that the mortality rate of Bioflash® on *An. stephensi* with factory recommended dose in both laboratory and semi-field conditions were higher than other tested doses. These results also indicate that with increasing concentration of Bioflash®, the efficiency of the applied dosages will not increase.

The efficacy of applied *Bacillus thuringiensis* (Bioflash®) formulation in laboratory condition against immature stages of *Anopheles* was higher that semi-field trial. The mortality rate of used bacterium in semi-filed condition was more than 21%. We can attribute these results to some important ecological and biological factors.

The effect of water volume on the efficiency of a biological agent, other symbiont and non-symbiont organisms in the habitat, different geographic conditions, survival ability of any

biologic factors, inappropriate formulation with breeding places conditions, and inaccuracy of formulation during the production process in the factory could be the most probable reasons of this inefficiency in field and semi-field conditions.

The findings of this study indicated a low efficacy of Bioflash®, granule formulation, at the recommended manufacturer's dosage, against *An. stephensi* larvae especially in semi-field conditions. These findings are consistent with various trials which have already been done in Iran (7).

According to the results of this study, the use of bacteria alone cannot be a useful and effective way to control the vector of malaria in Iran's geographic conditions. Therefore, this method can be used (in case of an appropriate efficacy of the tested formulation in field condition) as one of the constituent parts of Integrated Vector Management (IVM) program along with other recommended methods.

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