Original Article

Evaluation of the Time Course on the Effectiveness of WHO Standard Pyrethroid and Carbamate Impregnated Test Papers against *Anopheles stephensi*, the Main Malaria Vector in Iran

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Abstract

Background: *Anopheles stephensi* is a major vector of malaria in some parts of the world. A standard method for determining resistance in adult mosquito populations is the bioassay test recommended by the world health organization (WHO). The papers used in this method have an expiry date. This study aimed to determine the effectiveness of outdated susceptibility test papers for use in insecticide resistance monitoring programs.

Methods: Beech and Bandar Abbas strains of *An. stephensi* were reared in the insectary. Permethrin 0.75%, Deltamethrin 0.05%, and Bendiocarb 0.1% impregnated test papers prepared by Universiti Sains Malaysia were used. Probit analysis was used to analyze the results and prepare time-mortality regression lines of LT_{50} and LT_{90}.

Results: There was a difference in the mortality of both tested strains of *An. stephensi* was exposed to all tested insecticides. Both expired and not expired Permethrin and Deltamethrin papers induced 100% mortality at the diagnostic time (60min), but their insecticidal properties were reduced gradually in serial times. The highest efficacy of test papers was in the first trimester after the expiry date and decreased over time.

Conclusion: At the diagnostic time of 60 minutes, the mortality rate of both dated and expired papers was 100% in the pyrethroid insecticides, even three years after expiry dates, if stored in the package provided by the producer, in a refrigerator. This value was reduced to less than 100% in the expired papers of Bendiocarb comparing the dated papers that induced 100% mortality.

Keywords: Susceptibility test; *Anopheles stephensi*; Insecticide impregnated papers; Expiration date

Introduction

Malaria is an infectious disease and one of the major health problems worldwide. In 2021, estimated 247 million cases of malaria occurred worldwide, resulting in 619,000 deaths (1). The disease is caused by *Plasmodium* parasites and is transmitted to humans by the bites of *Anopheles* mosquitoes (2). One of the methods to prevent and fight this disease is to control vectors in different ways. Due to the importance of transmitting this disease in Iran, especially in the southern and southeastern regions, vector control operations are carried out regularly in these areas. In the global malaria strategy, the use of insecticides has maintained its special place in control operations. Following the increasing use of various pesticides and the emergence of the phenomenon of physiological and behavioral resistance of vectors, major problems

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have been created in malaria control programs.

One of the most important vectors of malaria in Iran is *Anopheles stephensi*, whose distribution is limited to the provinces of Sistan and Baluchestan, Hormozgan, Bushehr, Kerman, Fars, Khuzestan, Ilam, Lorestan, Kohgiluyeh and Boyer-Ahmad, and Kermanshah (3, 4). Extensive use of residual spraying to control malaria vectors has led to resistance of *An. stephensi* to DDT, Dieldrin, Malathion and other organophosphates, Bendiocarb, and some pyrethroids in the Middle East (5). The first report of resistance in *An. stephensi* was to DDT in 1957 in the south of the country (6). So, Dieldrin replaced DDT in the indoor residual spraying (IRS) program, but unfortunately, three years after the application of dieldrin, in 1960, cases of resistance to this insecticide were observed in the areas under the coverage of IRS (7). Malathion, Propoxur, Lambda-cyhalothrin, Deltamethrin, and Bendiocarb, are other insecticides used for IRS in Iran, respectively. Unfortunately, recent studies in the last decade show *An. stephensi* is resistant to all groups of insecticides in some parts of Iran (8–13).

Increasing resistance to insecticides is a complex and dynamic process and depends on many factors. Increasing the dose of insecticide is not recommended to maintain effectiveness because it creates environmental problems and adversely affects human life. The resistance gene in the vector population may also increase dramatically. Often, when the number of resistant insects in a vector population increases, the effectiveness of a particular insecticide decreases to the point that it must be replaced by another insecticide. Therefore, insecticide resistance management is a very important issue that should be considered in malaria control programs. Detection of insecticide resistance in natural vector populations is essential for malaria control. A standard method for determining resistance in adult mosquito populations is the bioassay test recommended by the World Health Organization (14). In the WHO standard method, mosquito specimens are exposed to a series of different insecticides using insecticide-impregnated papers at differential or diagnostic doses. This method has been widely used in this field and gives acceptable results in detecting insecticide resistance for monitoring purposes. The papers used in this method have an expiration date, so due to the customs consideration in some countries, it is a time-consuming process from the date they are produced until they reach the malaria vector control authorities; and may expire by the appropriate time for starting the susceptibility tests.

In 1957, during studies on the expiration date of insecticide-impregnated papers in susceptibility tests against *Aedes aegypti* using the WHO standard method, it was found no significant difference between the two groups of papers impregnated with DDT and Dieldrin with different ages after production (15). In reviewing previous research around the world, we found that no other study has been conducted in this field since that date. During these years, new insecticides have been introduced and used around the world to control malaria vectors. Insecticide-impregnated test papers have also changed a lot.

This study aimed to answer the question of whether the expiration date listed on the susceptibility test papers can be a deterrent to the use of these papers or influence their lethality.

Materials and Methods

Mosquito strains

We used a field strain of this mosquito collected from Hormoodar Village (56.32 ^{o}E, 27.31 ^{o}N), Bandar Abbas County, southern Iran, as well as the Beech susceptible laboratory strain. Phenotypic resistance profile of the wild population (Bandar Abbas) of *An. stephensi* has been identified in a previous study (9). The collected samples from the field were transferred to the insectary and reared to establish the laboratory colony. The rearing conditions were at 30±2 °C, 65±5% relative humidity, and a light to dark period of 12 to 12 hours (16).

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An artificial blood-feeding device with human whole blood was used to feed the adult female mosquitoes and this operation was repeated every three days (17). The colony was fed on a 10% sucrose solution.

Insecticides

Three insecticides that are used by the Iran national malaria control program, including Permethrin (in the long-lasting insecticide-treated nets (LLINs), Deltamethrin, and Bendiocarb (in IRS), were considered in this study. So, for this experiment, we used Deltamethrin 0.05%, Permethrin 0.75%, and Bendiocarb 0.1% impregnated papers prepared by the WHO collaboration center, Universiti Sains Malaysia (USM) (Table 1). Two series of each insecticide were used in this study with different impregnation and expiry dates. These papers were provided to us by the Center for Disease Management of the Ministry of Health and kept in the refrigerator under standard conditions.

Susceptibility tests

Tests started on 9 Dec 2020 and finished on 27 August 2021. Three to five-day-old nulliparous female mosquitoes fed with sucrose 10% were used in insecticide susceptibility tests, according to the WHO standard method and using standard impregnated papers (15). Each experiment for each tested time consisted of four replications and two controls, and in each replication, 25 adult mosquitoes were tested. The tests were performed at serial times of 7.5, 15, 30, and 60 minutes with the diagnostic dose of each insecticide. Shorter times were used as needed. With this method, we tested the efficacy of the test papers in serial time under 60 minutes to be able to calculate the LT_{50} and to compare the LT_{50s} between the different groups of test papers. According to the WHO instructions, each paper was used only 6 times (15). Then, the tested mosquitoes were kept in the insectary condition for 24 hours. The number of dead specimens was recorded at the end of the above time. If the mosquitoes in the control group had no mortality or less than 5%, the test was considered correct. If the control mosquitoes had a mortality of 5–20%, the results were corrected with the Abbott formula. Also, if the control mortality was more than 20%, the test was unacceptable and repeated (15).

Data analysis

Probit software (18) was used to analyze the results and to prepare a lethal time regression line. The results were displayed using descriptive tables and graphs. By comparing the mortality rate of the papers impregnated with a specific insecticide and its standard deviation with different expiration dates, the effect of time course on the lethality of these papers was investigated.

Results

Bendiocarb 0.1%

Beech laboratory and Bandar Abbas strains of *An. stephensi* were tested with the papers produced in Aug. 2017, expired in Aug. 2020, and tested in Feb. 2021. These tests were performed in five serial times (225, 450, 900, 1800, and 3600 sec). The regression line equations obtained from probit analysis were calculated as Y= 0.0007X+3.5474 and Y= 0.0007X+3.405 respectively (Fig. 1).

Also, the two strains were tested with the papers produced in Aug. 2019, expired in Aug. 2022, and tested in May 2021. These tests were performed with seven serial times (56, 112, 225, 450, 900, 1800, 3600 sec). The regression line equations obtained from probit analysis were calculated as Y= 0.0023X+ 4.1386 and Y= 0.002X+3.645 (Fig. 1). The LT_{50} and LT_{90} values for the two Bendiocarb papers are presented in Fig. 2.

Permethrin 0.75%

Beech laboratory and Bandar Abbas strains of *An. stephensi* were tested with the papers produced in Aug. 2017, expired in Aug. 2018, and tested in March 2021. These tests were

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performed in eight serial times (28, 56, 112, 225, 450, 900, 1800, and 3600 sec). The regression line equations obtained from probit analysis were calculated as Y= 0.0035X+ 4.5092 (Fig. 3) and Y= 0.0019X+4.6029 (Fig. 5), respectively.

Also, the two strains were tested with the papers produced in Aug. 2019 and expired in Aug. 2020. These tests were performed with 8 serial times (28, 56, 112, 225, 450, 900, 1800, 3600 sec), three, six, and nine months after expiration. For the Beech strain, the regression line equation obtained from probit analysis was calculated as Y= 0.0074X+4.4337, Y= 0.0036X+4.7234, and Y= 0.0032X+4.7962 for the 1st, 2nd, and 3rd trimesters, respectively (Fig. 3). The LT_{50} and LT_{90} values are presented in Fig. 4. The LT_{50} and LT_{90} values are presented in Fig. 4. For Bandar Abbas strain, the regression line equations for the first, second, and third trimesters were Y= 0.0088X+ 4.1856, Y= 0.0051X+4.4706, and Y= 0.0039X+4.6843, respectively (Fig. 5).

Deltamethrin 0.05%

Beech laboratory and Bandar Abbas strains of *An. stephensi* were tested with the papers produced in Aug. 2017, expired in Aug. 2018, and tested in March 2021. These tests were performed in eight serial times (28, 56, 112, 225, 450, 900, 1800, and 3600 sec). The regression line equations obtained from probit analysis were calculated as Y= 0.0015X+4.7598 (Fig. 6) and Y= 0.0038X+4.0065 (Fig. 7), respectively.

Also, the two strains were tested with the papers produced in Aug. 2019 and expired in Aug. 2020. These tests were performed with 8 serial times (28, 56, 112, 225, 450, 900, 1800, 3600 sec), three, six, and nine months after expiration. For the Beech strain, the regression line equation obtained from probit analysis was calculated as Y= 0.0031X+4.6574, Y= 0.0015X+5.1529, and Y= 0.0016X+5.0414 for the 1st, 2nd, and 3rd trimesters, respectively (Fig. 6). The LT_{50} and LT_{90} values are presented in Fig. 7. For Bandar Abbas strain, the regression line equations for the first, second, and third trimesters were Y= 0.0033X+4.3534, Y= 0.0017X+4.6184, and Y= 0.0008X+4.9212, respectively (Fig. 7). The LT_{50} and LT_{90} values are presented in Fig. 8.

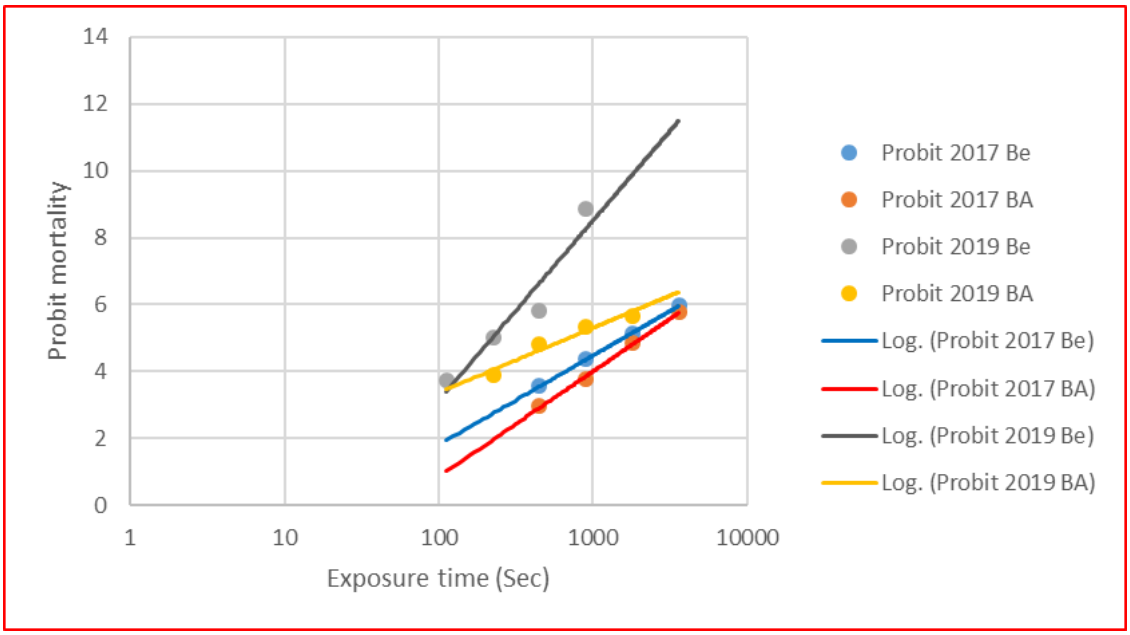

Fig. 1. Comparison of time-mortality regression lines due to exposure of *Anopheles stephensi* with Bendiocarb 0.1% with two different producing and expiration dates. Be= Beech strain, BA= Bandar Abbas strain

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Table 1. Specifications of insecticides used in this study

Insecticide	Impregnation date	Expiry date	Batch number	Control number
Deltamethrin 0.05%	August 2017	August 2018	DE 527	527
Permethrin 0.75%	August 2017	August 2018	PE 428	428
Deltamethrin 0.05%	August 2019	August 2020	DE 717	717
Permethrin 0.75%	August 2019	August 2020	PE 594	594
Bendiocarb 0.1%	August 2017	August 2020	BE 200	200
Bendiocarb 0.1%	August 2019	August 2022	BE 272	272

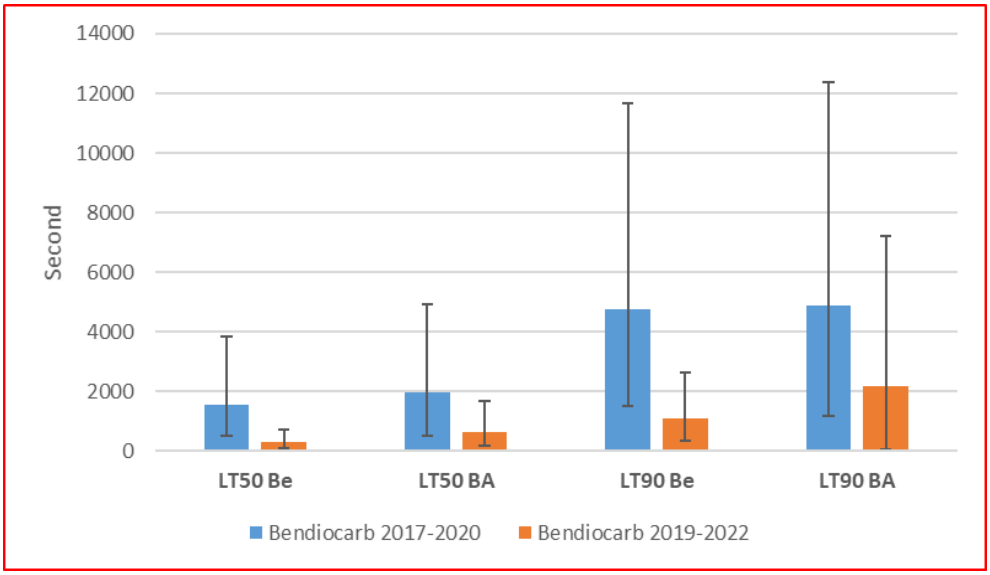

Fig. 2. Comparison of LT_{50} and LT_{90} values of two strains of *Anopheles stephensi* after exposure with Bendiocarb 0.1% with two different expiration dates. Be= Beech strain, BA= Bandar Abbas strain. Error bars show standard deviation (SD)

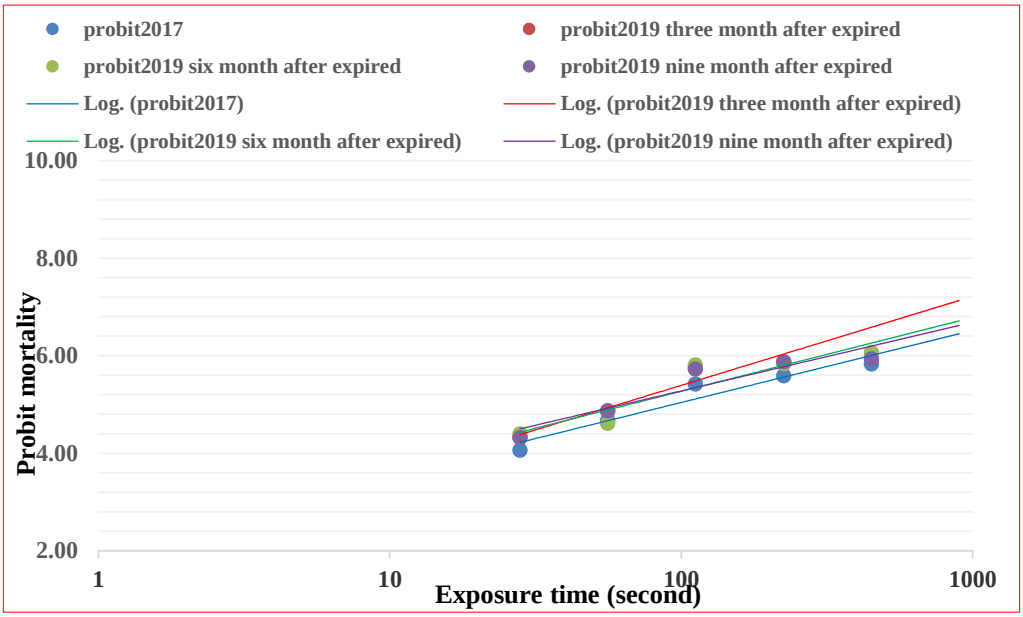

Fig. 3. Comparison of the regression lines of time-mortality due to exposure of *Anopheles stephensi* (Beech strain) to Permethrin 0.75% with two different production and expiration dates

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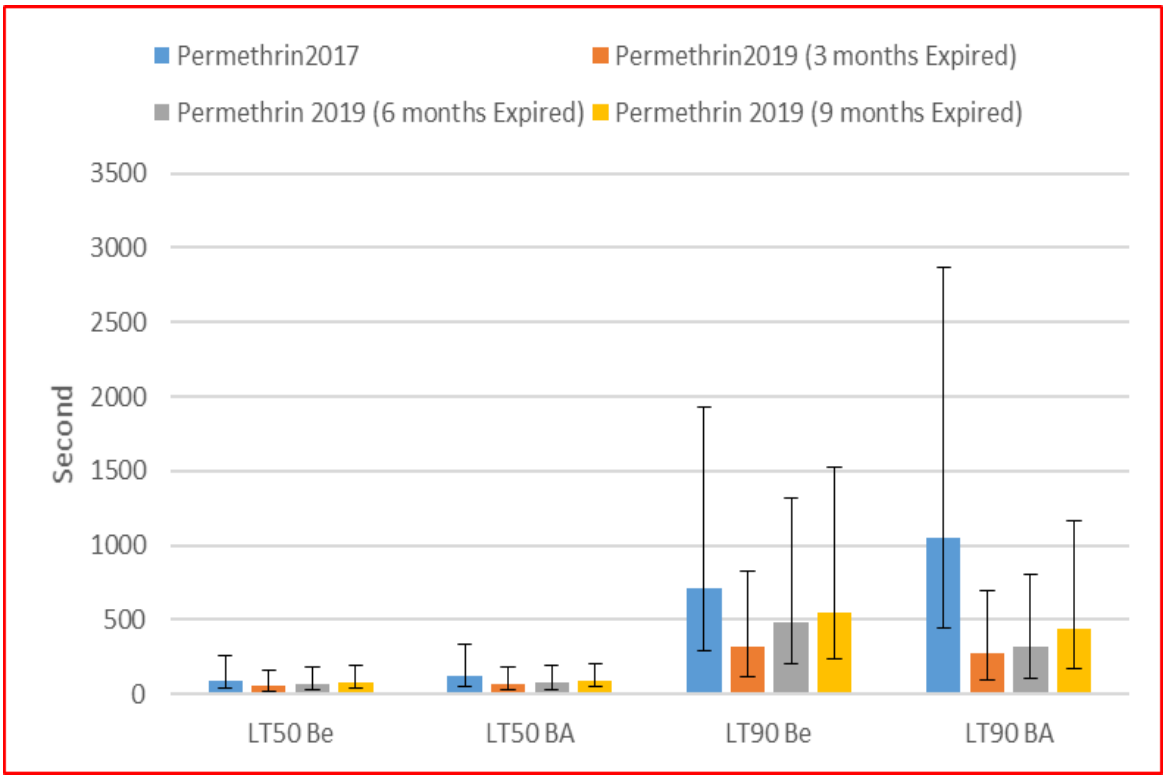

Fig. 4. Comparison of LT_{50} and LT_{90} values of two strains of *Anopheles stephensi* after exposure with Permethrin 0.75% with two different expiration dates. Be= Beech strain, BA= Bandar Abbas strain. Error bars show standard deviation (SD)

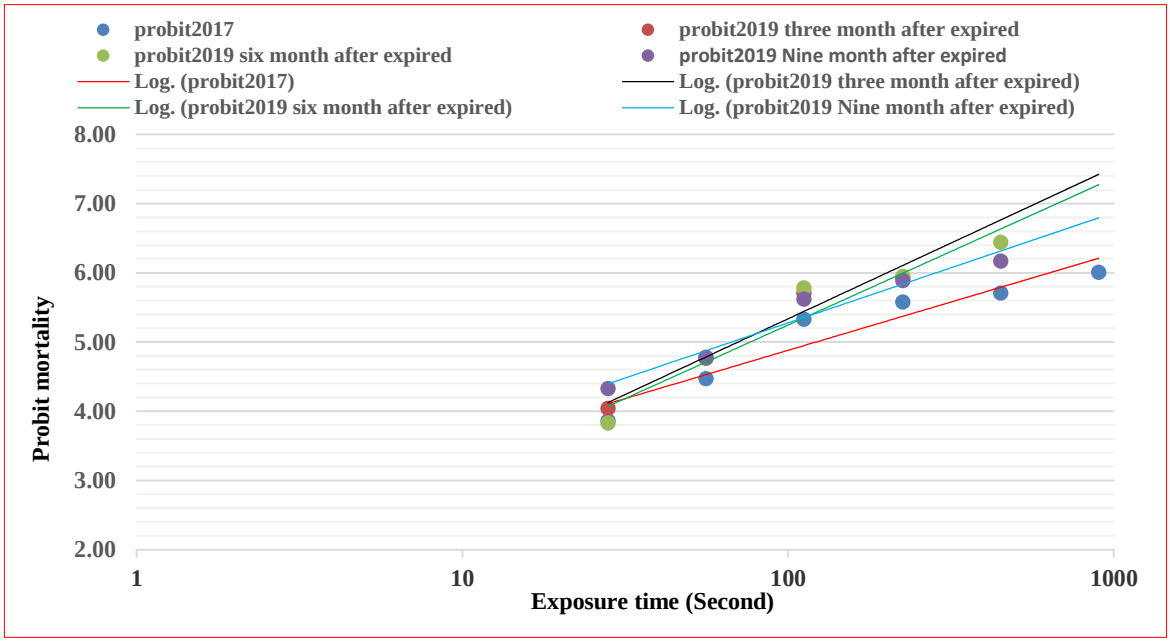

Fig. 5. Comparison of the regression lines of time-mortality due to exposure of *Anopheles stephensi* (Bandar Abbas strain) to Permethrin 0.75% with two different production and expiration dates

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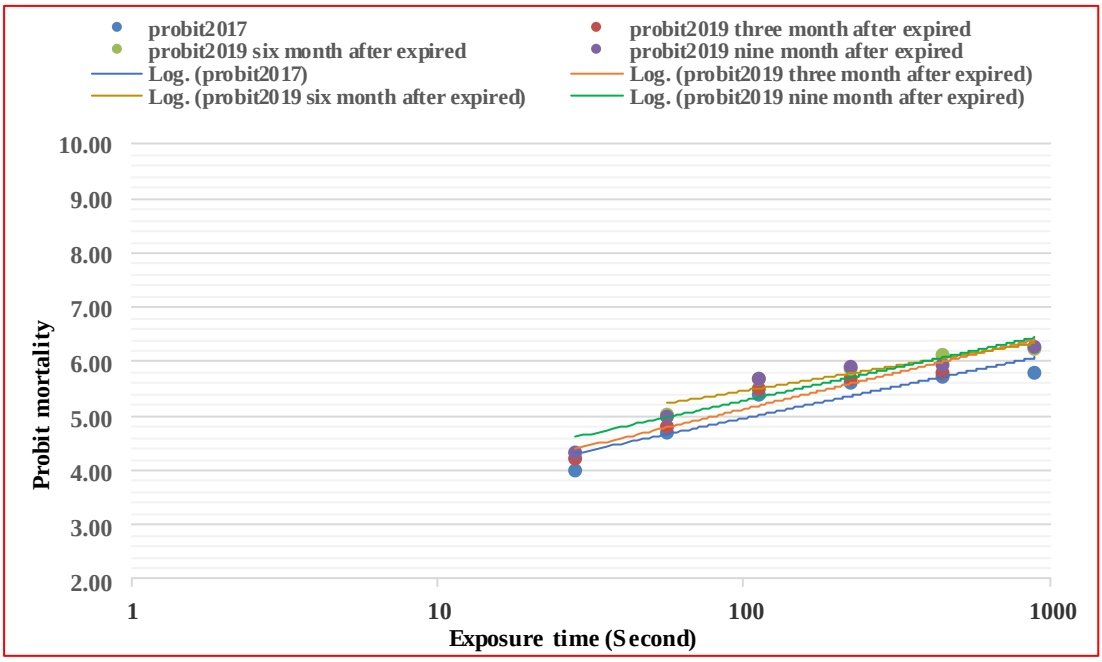

Fig. 6. Comparison of the regression lines of time-mortality due to exposure of *Anopheles stephensi* (Beech strain) to Deltamethrin 0.05% with two different production and expiration dates

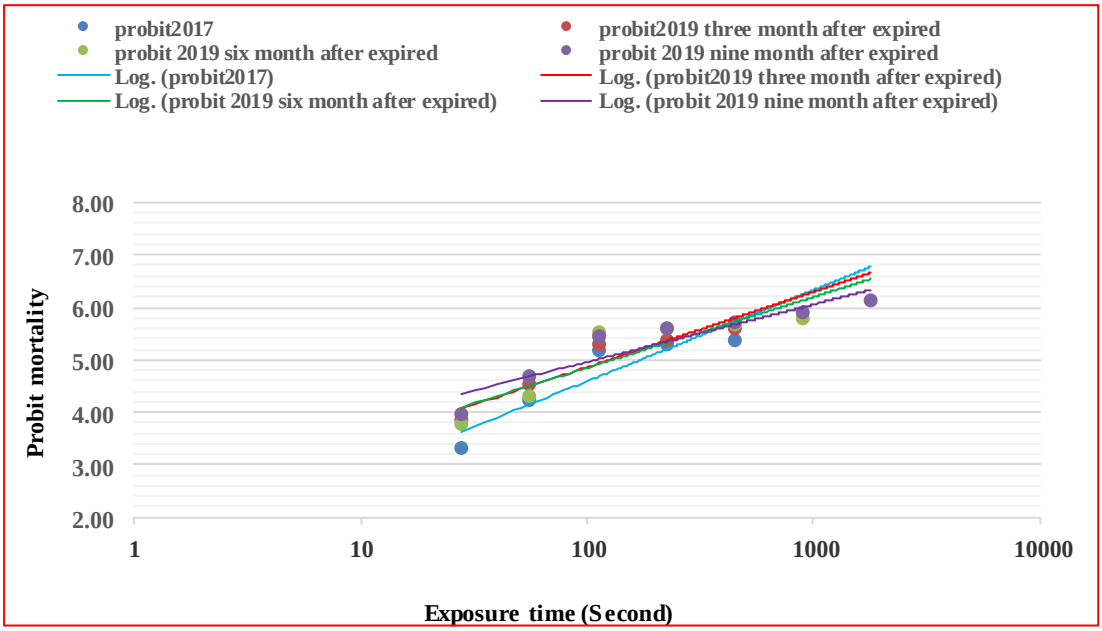

Fig. 7. Comparison of the regression lines of time-mortality due to exposure of *Anopheles stephensi* (Bandar Abbas strain) to Deltamethrin 0.05% with two different production and expiration dates

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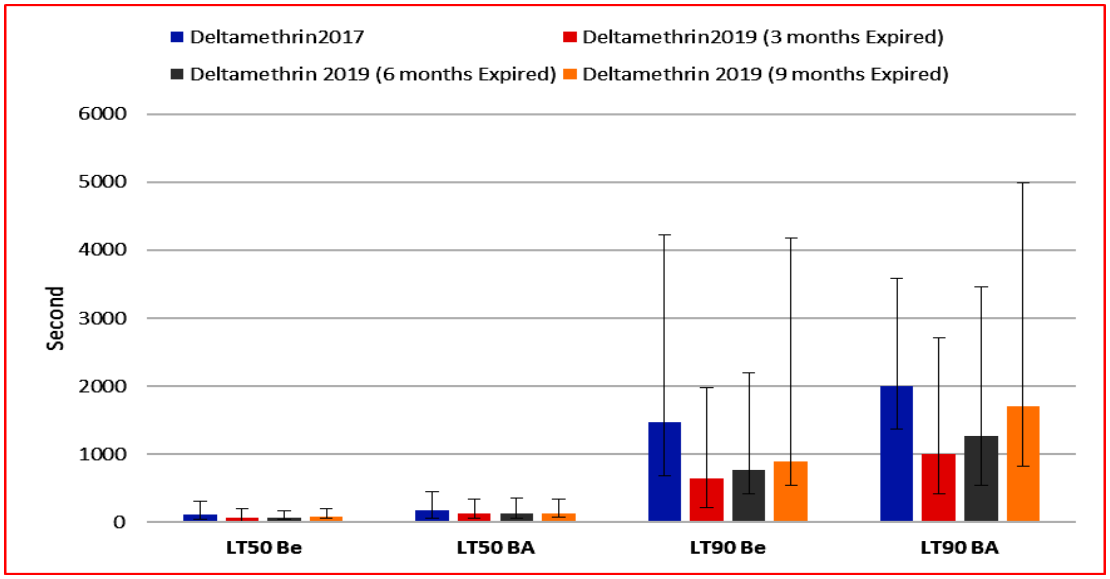

Fig. 8. Comparison of LT_{50} and LT_{90} values of two strains of *Anopheles stephensi* after exposure with Deltamethrin 0.05% with two different expiration dates. Be= Beech strain, BA= Bandar Abbas strain. Error bars show standard deviation (SD)

Discussion

Given that our study is the first in the world on susceptibility test papers produced by the WHO collaboration center in Malaysia (USM) and there are no similar studies for it, in this section, we discuss the insecticide used and the reasons for the decrease in the effectiveness of susceptibility test papers at different times after production. Although in a study conducted in the 1960s using DDT test papers against *Ae. aegypti*, each set of papers killed very similar proportions of the tested mosquitos (15). In this study, we found in all tests performed using deltamethrin 0.05% and permethrin 0.75% according to the standard method of the WHO in the diagnostic time of 60 minutes, in both dated and outdated papers, the mortality rate was found to be 100% in Beech and Bandar Abbas strains of *An. stephensi*.

Comparing the regression lines of time mortality obtained from probit analysis of Bendiocarb insecticide against Beech strain of *An. stephensi* with different expiration dates, it is clear that the insecticidal properties decreased one year after the expiration date of Bendiocarb 0.1% impregnated papers. Also, the values of LT_{50} and LT_{90} in expired papers have increased 5.4 and 4.4 times, respectively (Fig. 2). Also, in the tests performed with 2017–2020 Bendiocarb impregnated papers, the LT_{90} value is 3 times $LT_{50,}$ while for 2019–2022 Bendiocarb papers, the LT_{90} value is 3.7 times LT_{50}. Comparing the regression lines of time mortality obtained from probit analysis of Bendiocarb insecticide against Bandar Abbas strain of *An. stephensi* with different expiration dates, it is clear that the insecticidal properties decreased one year after the expiration date of Bendiocarb 0.1% impregnated papers. Also, the values of LT_{50} and LT_{90} in expired papers have increased by 3.2 and 2.2 times, respectively (Fig. 2). Also, in the tests performed with 2017–2020 Bendiocarb impregnated papers, the LT_{90} value is 2.5 times $LT_{50,}$ while for 2019–2022 Bendiocarb papers, the LT_{90} value is 3.5 times LT_{50} (Fig. 2).

Comparing the regression lines of time mortality obtained from probit analysis of Permethrin insecticide against Beech strain of *An.*

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stephensi with different expiry dates, the insecticidal properties decreased over time. Also, the LT_{50} and LT_{90} in the 2017–2018 papers have increased compared to the papers of 2019–2020 (1.5 times for LT_{50} and 2.3 times for LT_{90}). The results showed that in the third trimester (9 months after the expiration of 2019–2020 papers), the values of LT_{50} and LT_{90} have increased by 1.04 times for LT_{50} and 1.7 times for LT_{90} compared to the first trimester (Fig. 4). Comparing the regression lines of time mortality obtained from probit analysis of Permethrin insecticide against Bandar Abbas strain of *An. stephensi* with different expiry dates, the insecticidal properties decreased over time. Also, the LT_{50} and LT_{90} in the 2017–2018 papers have increased compared to the papers of 2019–2020 (2.1 times for LT_{50} and 6.3 times for LT_{90}). The results showed that in the third trimester (9 months after the expiry date of 2019–2020 papers), the values of LT_{50} and LT_{90} increased 1.7 times for LT_{50} and 3.8 times for LT_{90} compared to the first trimester (Fig. 4).

Comparing the regression lines of time mortality obtained from probit analysis of Deltamethrin insecticide against Beech strain of *An. stephensi* with different expiration dates, the insecticidal properties decreased over time. Also, the LT_{50} and LT_{90} in the 2017–2018 papers have increased compared to the papers of 2019–2020 (1.7 times for LT_{50} and 2.3 times for LT_{90}). The results showed that in the third trimester (9 months after the expiration of 2019–2020 papers), the values of LT_{50} and LT_{90} increased by 1.4 times for LT_{50} and 1.4 times for LT_{90} compared to the first trimester (Fig. 8). Compared the regression lines of time mortality obtained from probit analysis of Deltamethrin insecticide against Bandar Abbas strain of *An. stephensi* with different expiry dates, the insecticidal properties decreased over time. Also, the LT_{50} and LT_{90} in the 2017–2018 papers have increased compared to the papers of 2019–2020 (1.4 times for LT_{50} and 2 times for LT_{90}). The results showed that in the third trimester (9 months after the expiry date of 2019–2020 papers), the values of LT_{50} and LT_{90} increased 1.1 times for LT_{50} and 1.7 times for LT_{90} compared to the first trimester (Fig. 8).

The decrease in mortality and the increase in LT_{50} and LT_{90} are due to the decrease in the insecticidal properties of the insecticides used, which can be due to several reasons, including breaking and decomposing part of the insecticide or evaporating it later. This issue should be further investigated and the volume of insecticide residues on susceptibility test papers should be measured with special facilities and methods (19, 20). Also, another reason for the decrease in the quality of these papers over time can be related to the storage conditions and how these papers are stored. In the interval between tests, the papers should be stored in their original plastic box, to be sealed with adhesive tapes, and stored in the refrigerator at 4 °C or, if this is not possible, in a dark cupboard at room temperature. Papers stored at 4 °C should be exposed to room temperature before use. Insecticide-impregnated papers should never be exposed to direct sunlight (14).

In general, by comparing the mortality rates of two strains of Bandar Abbas and Beech at different serial times in the presence of Bendiocarb 0.1%, Permethrin 0.75%, and Deltamethrin 0.05%, we conclude that the susceptible laboratory strain in exposure to insecticide-impregnated papers is more sensitive because it had a higher mortality rate. The results of the most recent study in Bandar Abbas showed this species was resistant to Bendiocarb 0.1%, Permethrin 0.75%, and Deltamethrin 0.05% (9). The lower mortality rate in the Bandar Abbas strain could be due to the presence of resistance genes or metabolic mechanisms in the natural population of this strain because this strain was collected from the field and used after breeding in the insectary. Synergist bioassays on the DDT and Permethrin-resistant laboratory strains of *An. stephensi* from Iran indicated the metabolic resistance in this species (21). Another study in Iran confirmed that metabolic mechanisms play a critical role in the

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resistance of *An. stephensi* to Cyfluthrin, a pyrethroid insecticide (12).

It is recommended that additional studies be performed on insecticide residues and their metabolites on the WHO susceptibility test papers at different times after production. Due to the special conditions of the Covid-19 pandemic, access to papers with different production dates was not possible for this study. This is the limitation of this research.

Conclusions

At the diagnostic time of 60 minutes, the mortality rate of dated and expired papers was equal in the pyrethroid insecticides, even three years after expiry dates, if stored in their standard package in the refrigerator. About the Bendiocarb, the mortality rate of the tested mosquitoes was less than 100% in the expired test papers. Due to the high cost of test papers for the countries which are involved in malaria diseases, especially in the African region, the results of this experiment are interesting. By the way, to chemically analyze the residual amount of insecticides or their metabolites on the papers using HPLC or GC is suggested to have an idea about the active ingredient remaining on the papers on different dates.

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Ethical considerations

This research has been registered with the ethics code IR.TUMS.SPH.REC.1399.175 in the Ethics Committee of the School of Public Health, Tehran University of Medical Sciences.

Conflict of interest statement

The authors declare there is no conflict of interests.

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