



Molecular Detection of Common *Plasmodium* Species in Malaria Vectors in Villages of Chabahar and Konarak, Iran

Hadi Mirahmadi ^{1,2}, Mansour Rahmati-Balaghaleh ^{1,2}, Soudabeh Etemadi ^{1,2}, Samaneh Abdolahi Khabisi ^{1,2}, Seyed Mehdi Tabatabaei ³, Ahmad Mehravaran ^{1,2,*} and Mehdi Shahraki ^{2,**}

¹Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis Institute, Zahedan University of Medical Sciences, Zahedan, Iran

²Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran

³Department of Statistic and Epidemiology, Zahedan University of Medical Science, Iran

*Corresponding author: Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis Institute, Zahedan University of Medical Sciences, Zahedan, Iran. Email: ahmadmehravaran55@gmail.com

**Corresponding author: Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran. Email: mehdishahraki941120@gmail.com

Received 2020 May 26; Revised 2020 November 14; Accepted 2021 January 07.

Abstract

Background: Malaria is one of the most serious parasitic diseases and the major health issue in tropical and subtropical countries.

Objectives: This study aimed to investigate the type of infection of female Anopheles mosquitoes to sporozoites of *Plasmodium* species (*Plasmodium vivax* and *Plasmodium falciparum*), using nested polymerase chain reaction (PCR) in southeastern Iran.

Methods: To determine the infections and *Plasmodium* species using the nested PCR in Sistan and Baluchestan Province (southeastern Iran), 360 female Anopheles mosquitoes were collected from the control villages of Chabahar and Konarak counties and examined by entomology check stations affiliated with Zahedan University of Medical Sciences in the second half of 2018. The mosquitoes were caught manually from indoor places (humans and animals) as well as natural and artificial sites, i.e., pit-shelters. After DNA extraction, the molecular analysis was performed by nested PCR.

Results: Of 200 species collected from Daregas, Owraqi, and Bahu Kalat in Chabahar, four *Anopheles stephensi* (2%) were infected with *P. vivax*, and of 160 samples collected from the rural districts of Konarak, including Zarabad, Heyan, Islamabad, and Joholu, two *An. stephensi* (1.25%) were infected with *P. falciparum*. In this study, no mixed infection of *Plasmodium vivax* and *Plasmodium falciparum* were observed.

Conclusions: The present study showed that the only species infected with *Plasmodium* in the aforementioned regions was *An. stephensi*, which was the most frequent species of Anopheles among other species as well. The nested PCR could be the most suitable method for detecting *Plasmodium* parasites in malaria vectors, among other diagnostic methods.

Keywords: Malaria Vectors, Nested PCR, *Plasmodium* Species

1. Background

Malaria is an infectious disease in developing countries, which is characterized by chills (shivering), fever, and sometimes severe and fatal complications. It is globally important in terms of health, being one of the six important diseases in the World Health Organization (WHO) programs for tropical and subtropical regions (1, 2). According to the latest WHO report released in 2016, despite various malaria control and anti-malaria programs, approximately 216 million cases of malaria have been reported from 91 countries, which has been about 5 million cases more than the figure reported in 2015. The number of fatalities has been reported to have been 445,000 persons worldwide (3). Iran is one of the recognized malaria-

endemic countries in the eastern Mediterranean region. The southern and southeastern regions of Iran are malaria-endemic, and the two southeastern provinces of Sistan and Baluchestan and Hormozgan, as well as Jiroft and Kohnuj counties in Kerman province, have the highest rate of malaria cases across the country (4, 5). The primary goal of the malaria elimination program on the Horizon of Iran 1404 (2025) is to stop the local transmission of the disease and turn Iran into a malaria-free region (6, 7).

The basic strategy in the global malaria control program is to control Anopheles mosquitoes, which has been proved as one of the best and most effective methods of stopping and reducing the malaria transmission rate around the world. The awareness of the composition of Anopheles species and the number of sporozoites in their

salivary glands, which determines the role of each species in the transmission of the disease, are key epidemiological indicators required to plan for malaria control and prevention methods commensurate with local conditions (8, 9).

Various biochemical, molecular, and immunological methods, such as isoenzyme electrophoresis, polymerase chain reaction (PCR), and monoclonal antibodies, are commonly used to diagnose *Plasmodium* in mosquitoes (10, 11). One of the most common methods for detecting malaria parasites in Anopheles mosquitoes is the dissection of salivary glands in captured female Anopheles, using stereomicroscope and light microscopy. However, this method is quite laborious, time-consuming, and of a low sensitivity level (10, 12). Among many methods proposed as alternatives to the microscopic detection of parasites in mosquitoes, nested PCR using 4 primers for the 18S subunit ribosomal ribonucleic acid (18SsrRNA) gene was introduced as a sensitive method with high specificity for the detection of *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malaria*, and *P. ovale*) (13). The nested PCR assay is highly sensitive and highly specific in malaria detection, which is capable of detecting 1-10 parasites per microliter (μL) of blood and is five times stronger than the microscopic detection power of Giemsa-stained spreads. Apart from detecting parasite species, it is also capable of detecting mixed infections. In addition, the high sensitivity and specificity of this method indicate its importance in the diagnosis of low-parasite cases and asymptomatic malaria. The nested PCR is a very suitable method for the detection of malaria in regions of a low transmission rate, which could be used to monitor drug resistance. Many samples could also be tested concurrent with using this technique (10).

In southern and southeastern Iran, due to the relatively favorable climatic and geographical conditions as well as its proximity to Pakistan, malaria is a major public health problem. Sistan and Baluchestan province, in southeastern Iran, has a long common border with Pakistan and has the highest incidence of malaria (14).

2. Objectives

This study aimed to investigate the rate and type of the infection of female Anopheles mosquitoes with sporozoites of the malaria parasite using the nested PCR in Chabahar and Konarak counties of Sistan and Baluchestan province.

3. Methods

3.1. The Study Region

Sistan and Baluchestan province is located in southeastern Iran, with Chabahar and Konarak counties located

in the southeast of the province. Chabahar has three urban centers, including Chabahar, Negour, and Polan (Figure 1). It also has three districts, including the central district, Dashtiari, and Polan. In addition, it has six rural districts, including Pirsohrab, Kambel-e Soleyman, Nowbandian-e Payyn, Bahoukalat, Sand-e-Mirsuban, and Polan, with 446 inhabited villages. Konarak has two urban districts, including Konark and Zarabad, and four rural districts, namely Zarabad-e Sharqhi, Zarabad-e Gharbi (Caravan), Kahir, and Jahliyan. This province is located on the northern coast of the Oman Sea, where the warm and humid climate of the region is suitable for Anopheles species, which has made various Anopheles species be the main vector responsible for malaria transmission in southeastern Iran.

3.2. Sampling

This cross-sectional descriptive study was conducted in the second half of 2018 in the high-risk regions of malaria transmission in Sistan and Baluchestan province, i.e., Chabahar and Konarak. The disease in these regions has two peaks, from late April to early June and from August to late November, with the second peak being more severe. The districts surveyed in Chabahar included Daregas, Owraiki, and Bahu Kalat. In addition, the regions studied in Konarak were Zarabad, Heyan, Islamabad, and Joholou (Figure 1). The villages selected were sampled once every 15 days, and a total of 360 blood-fed Anopheles mosquitoes were hunted manually from indoor places (humans and animals) as well as natural and artificial sites (pit-shelters) by suction or aspirator tubes. All specimens caught by aspirators were killed by small cotton pads placed on plastic cups. Just after the samples were collected, they were immersed in tubes containing 70% alcohol and transferred to medical centers in Chabahar and Konarak. Using high magnification entomology loops, the blood-fed Anopheles were isolated. Next, they were identified and separated using the Iranian Adult Anopheles Recognition Key (15). The separated Anopheles samples were transferred into 1.5-ml tubes, and after writing specifications on the tubes, they were stored at -20°C until DNA extraction.

3.3. DNA Extraction

DNA extraction was conducted using DyanBio (DyanBio™ Boold/Tissue DNA Extraction Mini Kit) according to the instructions. The solution under the DNA column was kept at -20°C until PCR assessment.

3.4. Nested PCR

Nested PCR was performed with four pairs of primers and cycling conditions as described by Ebrahimzadeh et al. (16). The concentration of the final solution was

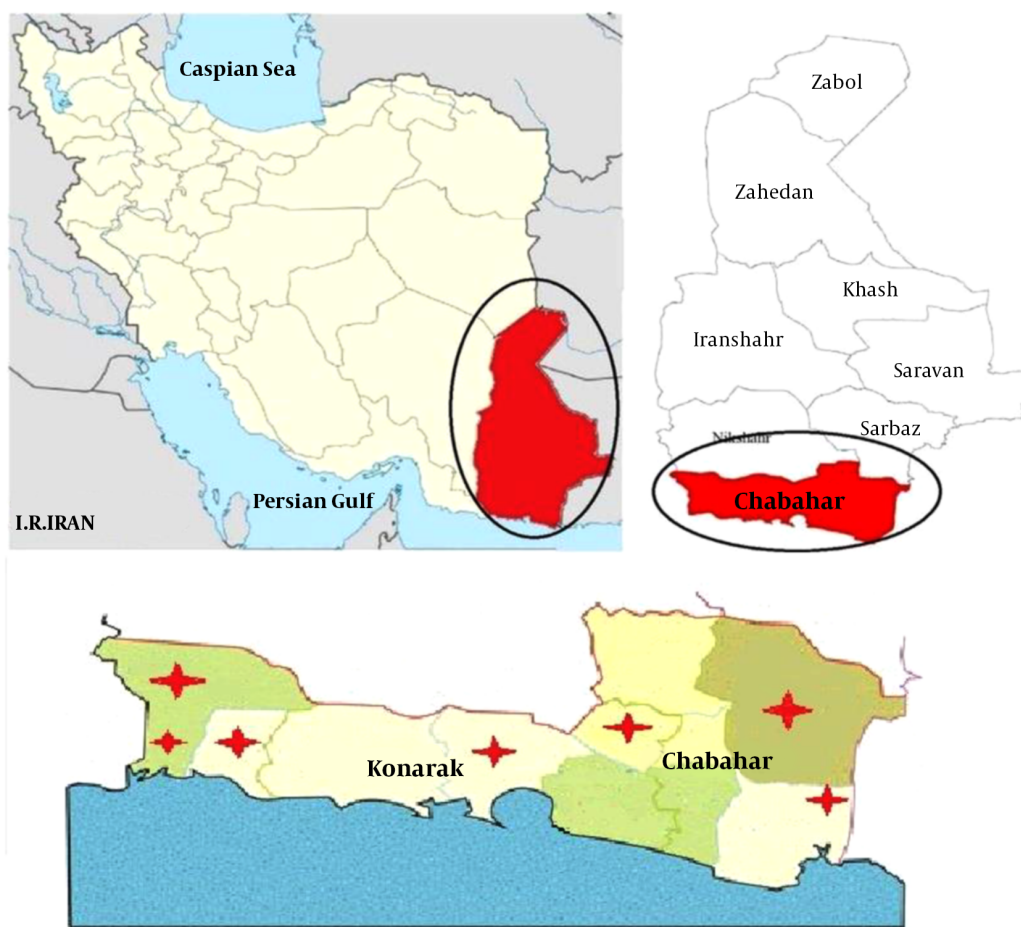


Figure 1. Sampling points of Chabahar and Konarak regions in Sistan and Baluchestan province of Iran, 200 samples (Daregas, Owraki, and Bahu Kalat in Chabahar), 160 samples rural districts of Konarak (Zarabad, Heyan, Islamabad, and Joholu).

20 Pmol/ μ L. The primers were kept at -20°C . DNA samples were processed by PCR to amplify species-specific sequences of 18srRNA genes of *P. vivax* and *P. falciparum*. The first PCR utilized rplu 5 and rplu 6 primers, and the second PCR used rFAL.1, rFAL.2, rVIV.1 and rVIV.2 primers (17). PCR products of the second round of the PCR were loaded onto a 2% agarose gel, and the results were compared with standard band markers of *P. vivax*, 120 bp, and *P. falciparum*, 205 bp (Figure 2).

4. Results

A total of 360 female Anopheles mosquitoes were captured. Of 200 samples collected from Daregas, Owraki, and Bahu Kalat in Chabahar, four *An. stephensi* (2%) were infected with *P. vivax* that positive cases were taken from Daregas (Two positive cases) and Bahu Kalat villages (Two

positive cases). In addition, of 160 samples collected from the rural districts of Konarak, including Zarabad, Heyan, Islamabad, and Joholu, two *An. stephensi* (1.25%) were determined to be infected with *P. falciparum* that positive cases were taken from Zarabad (Table 1). In total, 1.66% of the vectors of *An. stephensi* were infected with the *Plasmodium* parasite.

5. Discussion

Malaria is still a major health and medical problem in southeastern Iran, especially in Sistan and Baluchestan province. It plays an important role in restricting the economic, social, and economic development of this region and wastes a great deal of capital, labor, time, and energy annually. In this study conducted in two malaria-endemic regions of Sistan and Baluchestan province (southeast-

Table 1. Anopheles Collection Areas in Chabahar and Konarak in 2018

| Sample Collection Areas | <i>An. stephensi</i> (%) | <i>An. culicifacies</i> (%) | <i>An. d'thali</i> (%) | <i>An. polacrimus</i> (%) | Total |
|-------------------------|--------------------------|-----------------------------|------------------------|---------------------------|-------|
| Daregas | 50 (50) | 44 (44) | 5 (5) | 1 (1) | 100 |
| Bahu Kalat | 24 (48) | 17 (34) | 7 (14) | 2 (4) | 50 |
| Owraki | 33 (66) | 15 (30) | 2 (4) | 0 | 50 |
| Zarabad | 23 (40.35) | 23 (40.35) | 8 (14.03) | 3 (5.27) | 57 |
| Heyan | 15 (50) | 8 (26.67) | 7 (23.33) | 0 | 30 |
| Islamabad | 16 (45.71) | 13 (37.14) | 5 (14.3) | 1 (2.85) | 35 |
| Joholou | 28 (73.68) | 10 (23.62) | 0 | 0 | 38 |

**Figure 2.** Electrophoresis of the 18srRNA gene nested PCR products on a 2% agarose gel. Lane 1: DNA marker 100 bp, Lane 2: Negative control, Lanes 3,4: Negative sample, Lane 5: control positive *P. vivax* (120 bp), Lane 6: control positive *P. falciparum* (205 bp), Lane 7: *P. falciparum* (205 bp), Lanes 8, 9 and 10: *P. vivax*.

ern Iran), a total of 360 female Anopheles mosquitoes belonged to four species, including *An. stephensi*, *An. culicifacies*, *An. fluviatilis*, and *An. d'thali* were caught. In a study aimed at identifying malaria vectors in high-risk regions of malaria transmission in Hormozgan province (Iran), Azizi et al. (2011) reported that adult anopheles belonged to the eight species of *An. stephensi*, *An. moghulensis*, *An. d'thali*, *sergeanti*, *An. culicifacies*, *An. fluviatilis*, *An. superpictus*, and *An. trochidae* (18). A study conducted in Fars province (Iran) by Kalantari et al. (2017) showed that the 1,073 anopheles caught belonged to the four species of *An. d'thali*, *An. superpictus*, *An. acharovi*, and *An. stephensi* (19). Therefore, the diversity of Anopheles species in Iran is created by varied ecological, climatic, altitude, and atmospheric conditions of different geographical regions.

In the present study, 200 Anopheles mosquitoes collected from the regions studied in Chabahar were determined to be the dominant species of *An. stephensi*. In addition, *An. culicifacies*, *An. polacrimus*, and *An. d'thali* were among the other important species found in this area. According to the findings, four species of Anopheles

mosquitoes (2%) caught from Daregas and Bahu Kalat villages were infected with *P. vivax*. This finding is further supported by the fact that the predominant malaria species in Iran is *P. vivax* (20), and that 2% of mosquitoes are infected in Chabahar. Similar studies (Mirahmadi et al., 2019; Asmar et al., 2005; Oshaghi et al., 2003) conducted in malaria-endemic regions in Iran have shown that the predominant species causing malaria is Anopheles *P. vivax* (21-23). In the same vein, Guo Yu et al. (2013) and Kyu Sik Chang et al. (2016) performed molecular analyses of malaria vectors in China and South Korea, respectively, and reported that the predominant type of *P. vivax* malaria and the dominant Anopheles malaria were minimus and kolini, respectively (24, 25).

In the present study, of 160 species collected from the studied regions of Konarak county, *An. stephensi* was the dominant species followed by *An. culicifacies* and *An. d'thali* as the second and third most dominant species, respectively. It was also found out that the two species of *An. stephensi* mosquitoes (1.25%) captured from Zarrabad village were infected with *P. falciparum*. This case of infection has probably been due to the illegal entry of Afghan and Pakistani immigrants, who have been working on banana, mango, lotus, and date farms, and have been traveling to Afghanistan and Pakistan as the countries with the highest risk of malaria transmission (21, 26). In addition, the studies conducted by Nejati et al. (2012) in the city of Konarak, Raeesi et al. (2009) in the endemic regions of Iran, and Podat et al. (2006) in Bandar Abbas showed that essential malaria changes and the increased incidence of the disease depend on the presence of aliens (26-28).

Results from the analysis of the variety of mosquito species showed that *An. stephensi* was the most frequent species in Chabahar and Konarak. The similar coastal climates of these two counties are the main reason for the similar distribution of this species. *An. stephensi* appears to be the major vector of malaria in urban, lowland, and coastal regions (29, 30).

A study by Kalantari et al. (2017) in Fars province, Iran

showed that *An. d'thali* was the most frequent species, and no infection with *Plasmodium* parasite was reported in mosquitoes using nested PCR. In the present study, a total of 1.66% of *An. stephensi* were infected with the *Plasmodium* parasite because Sistan and Baluchestan province is one of the malaria-endemic regions. In addition, the inflection of *An. stephensi* with *Plasmodium* parasite could be attributed to the differences in the endemic and ecological conditions of this province with those of Fars province (19). The study by Gonzalez et al. (2017) in northern Kenya using nested PCR showed that *Anopheles albuminuous* was the most frequent species, which was infected with *P. vivax* and *P. falciparum* (31). In the same vein, Nepomichene (2015) conducted a study in Madagascar using nested PCR assay and showed that 9.5% of *An. castaneus* were infected with *P. vivax* and *P. falciparum* (32). Ashwani Kumar et al. (2016), in a study in western India using nested PCR assay, found out that 2.8% of *An. subpictus* were infected with *P. vivax* and *P. falciparum* (33). A study was conducted by Vu Duc Chinh et al. (2019) in Vietnam using nested PCR assay on infectious cases in captured *Anopheles*. According to the results of this study, *Anopheles inui* was the dominant species in the region infected with *P. falciparum*, *P. vivax*, *P. cynomolgi*, and *P. knowlesi* (34).

According to the results of the four studies reviewed above, the prevalence of different *Plasmodium* and *Anopheles* species in different regions of the world is affected by many factors, among which climate changes and ecological conditions are the most effective ones. Therefore, the results of the present study concerning the prevalence of *Plasmodium* and *Anopheles* species are different from those of the above-mentioned ones.

In this study, a nested PCR assay was used, which is more sensitive than the conventional PCR. In addition, apart from detecting parasite genus and species, it is capable of detecting mixed infections. The 18srRNA gene was used for this purpose. This nucleotide sequence is fixed in eukaryotic organisms, especially in the parts involved in the formation of the second structure of the molecule. Therefore, oligonucleotide primers specifically binding to the protected part of the 18srRNA of genus *Plasmodium* and also uniquely binding to a part of the 18srRNA gene of each *Plasmodium* parasite species could be developed and used for identifying *Plasmodium* species. The present study showed that nested PCR assay could be easily used for a large number of samples. This technique does not require the dissection of *Anopheles*' salivary glands; in addition, dried and old specimens can be examined using this method. Therefore, there is no need for equipping laboratories and dispatching personnel to malaria-endemic regions; hence, specimens collected in the region could be analyzed and tested in central laboratories (23, 30).

According to epidemiological studies, Iranian malaria species, unlike those in African and other countries, vary according to climatic conditions and are almost eliminated (17, 35). Since the malaria-endemic regions of Iran are undergoing the process of malaria elimination, one of the most effective ways of monitoring the above-mentioned program is to monitor the infection status in female *Anopheles* mosquitoes. In contrast to the past studies, the results of the present study showed that there are some infection cases in malaria-carrying mosquitoes in Chabahar and Konarak, so it seems that the elimination of malaria by 2026 will require further studies, investigations, and arrangements.

In the present study, the species infected with the *Plasmodium* parasite was the *An. stephensi* mosquitoes, which is in contrast to other studies in the southeastern region. This may be due to annual spraying (twice a year) of mosquito, problems with mosquito trapping, and malaria elimination program in Iran. Also, *An. stephensi* has a higher chance of coming into contact with humans due to being domesticated, and their presence in domestic and foreign places is considered a warning sign for the outbreak of malaria. On the other hand, the vector control program is planned based on the duration of seasonal activity of *Anopheles* mosquitoes and the trend of population changes (36, 37).

Pakistan is adjacent to Iran's Sistan, and Baluchestan Province, which according to the WHO, is one of the six countries in the Eastern Mediterranean region with a high rate of malaria transmission and about 100% of the population lives at risk, and the majority of malaria species are imported from Iran. Studies in Pakistan in 2018 show that 24% of malaria *falciparum* and 10% mixed infection by these two species *P. falciparum* and *P. vivax*, and may be due to the contrast between the percentage of infection reported in the carrier and the prevailing percentage of *P. vivax* in Iran (38).

5.1. Conclusions

The present study showed that the species infected with *Plasmodium* parasites in the studied regions was *An. stephensi*, which is the most frequent species among other species. The nested PCR may be a better method for detecting *Plasmodium* parasites in malaria vectors than other diagnostic methods.

Acknowledgments

The authors would like to thank Dr. Salimi from Zahedan University of Medical Sciences for kindly cooperation. This work is a part of M. Sc. thesis of Mahdi Shahraki,

which was financially supported by a Grant (440) from Zahedan University of Medical Sciences, which is fully appreciated

Footnotes

Authors' Contribution: H. Mirahmadi and A. Mehravaran conceived the study; M. Shahraki performed the experiments; M. Rahmati-Balaghaleh and S. Etemadi wrote the manuscript; Seyed Mehdi Tabatabaei and Samaneh Abdolahi Khabisi critically revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interests: The authors declare that they have no conflict of interest.

Ethical Approval: The study protocol was approved by the Research Ethics Committee of Zahedan University of Medical Sciences, Zahedan, Iran (IR.Zaums.fm.REC.1395.440).

Funding/Support: This work is a part of M. Sc. thesis of Mehdi Shahraki that was financially supported by a Grant (440) from Zahedan University of Medical Sciences.

References

- World Health Organization. *WHO informal consultation on fever management in peripheral health care settings: a global review of evidence and practice*. Geneva, Switzerland: WHO; 2013.
- World Health Organization. *World malaria report 2015*. WHO; 2016. 4 p. Contract No.: 9789241565158.
- Reiter P. Global warming and malaria: knowing the horse before hitching the cart. *Malar J*. 2008;7 **Suppl 1**:S3. doi: [10.1186/1475-2875-7-S1-S3](https://doi.org/10.1186/1475-2875-7-S1-S3). [PubMed: [19091037](https://pubmed.ncbi.nlm.nih.gov/19091037/)]. [PubMed Central: [PMC2604878](https://pubmed.ncbi.nlm.nih.gov/PMC2604878/)].
- Beljaev AE. [The malaria situation in the WHO eastern Mediterranean region]. *Med Parazitol (Mosk)*. 2000;(2):12-5. [PubMed: [10900909](https://pubmed.ncbi.nlm.nih.gov/10900909/)].
- Soleimani-Ahmadi M, Vatandoost H, Zare M, Turki H, Alizadeh A. Topographical distribution of anopheline mosquitoes in an area under elimination programme in the south of Iran. *Malar J*. 2015;14:262. doi: [10.1186/s12936-015-0771-7](https://doi.org/10.1186/s12936-015-0771-7). [PubMed: [26148647](https://pubmed.ncbi.nlm.nih.gov/26148647/)]. [PubMed Central: [PMC4491864](https://pubmed.ncbi.nlm.nih.gov/PMC4491864/)].
- Soleimani-Ahmadi M, Vatandoost H, Shaeghi M, Raeisi A, Abedi F, Eshraghian MR, et al. Field evaluation of permethrin long-lasting insecticide treated nets (Olyset((R))) for malaria control in an endemic area, southeast of Iran. *Acta Trop*. 2012;123(3):146-53. doi: [10.1016/j.actatropica.2012.04.004](https://doi.org/10.1016/j.actatropica.2012.04.004). [PubMed: [22579798](https://pubmed.ncbi.nlm.nih.gov/22579798/)].
- Akbari H, Majdzadeh R, Rahimi Foroushani A, Raeisi A. Timeliness of malaria surveillance system in Iran. *Iran J Public Health*. 2013;42(1):39-47. [PubMed: [23515191](https://pubmed.ncbi.nlm.nih.gov/23515191/)]. [PubMed Central: [PMC3595634](https://pubmed.ncbi.nlm.nih.gov/PMC3595634/)].
- Raghavendra K, Barik TK, Reddy BP, Sharma P, Dash AP. Malaria vector control: from past to future. *Parasitol Res*. 2011;108(4):757-79. doi: [10.1007/s00436-010-2232-0](https://doi.org/10.1007/s00436-010-2232-0). [PubMed: [21229263](https://pubmed.ncbi.nlm.nih.gov/21229263/)].
- Soltani A, Vatandoost H, Jabbari H, Mesdaghinia AR, Mahvi AH, Younesian M, et al. Field efficacy of expanded polystyrene and shredded waste polystyrene beads for mosquito control in artificial pools and field trials, Islamic Republic of Iran. *EMHJ-East Mediterr Health J*. 2012;18(10):1042-8. doi: [10.26719/2012.18.10.1042](https://doi.org/10.26719/2012.18.10.1042). [PubMed: [23301359](https://pubmed.ncbi.nlm.nih.gov/23301359/)].
- Mohammadzadeh T, Hatam G, Kalantari M, Sarkari B, Motazedian MH, Sadjjadi SM, et al. Molecular and microscopic-based characterization of plasmodium spp. in fars and hormozgan provinces, South of Iran. *J Trop Med*. 2014;2014:935469. doi: [10.1155/2014/935469](https://doi.org/10.1155/2014/935469). [PubMed: [24799917](https://pubmed.ncbi.nlm.nih.gov/24799917/)]. [PubMed Central: [PMC3988939](https://pubmed.ncbi.nlm.nih.gov/PMC3988939/)].
- Snounou G, Viriyakosol S, Xin Ping Z, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. *Molecular and Biochemical Parasitology*. 1993;61(2):315-20. doi: [10.1016/0166-6851\(93\)90077-b](https://doi.org/10.1016/0166-6851(93)90077-b).
- Arez AP, Lopes D, Pinto J, Franco AS, Snounou G, do Rosario VE. Plasmodium sp.: optimal protocols for PCR detection of low parasite numbers from mosquito (Anopheles sp.) samples. *Exp Parasitol*. 2000;94(4):269-72. doi: [10.1006/expr.2000.4496](https://doi.org/10.1006/expr.2000.4496). [PubMed: [10831396](https://pubmed.ncbi.nlm.nih.gov/10831396/)].
- Fuehrer HP, Noedl H. Recent advances in detection of Plasmodium ovale: implications of separation into the two species Plasmodium ovale wallikeri and Plasmodium ovale curtisi. *J Clin Microbiol*. 2014;52(2):387-91. doi: [10.1128/JCM.02760-13](https://doi.org/10.1128/JCM.02760-13). [PubMed: [24478466](https://pubmed.ncbi.nlm.nih.gov/24478466/)]. [PubMed Central: [PMC391344](https://pubmed.ncbi.nlm.nih.gov/PMC391344/)].
- Halimi M, Farajzadeh M, Delavari M, Takhtardeshir A, Moradi A. Modelling spatial relationship between climatic conditions and annual parasite incidence of malaria in southern part of Sistan&Balouchistan Province of Iran using spatial statistic models. *Pac J Trop Dis*. 2014;4(Suppl 1):S167-72. doi: [10.1016/S2222-1808\(14\)60434-5](https://doi.org/10.1016/S2222-1808(14)60434-5).
- Azari-Hamidian S, Harbach RE. Keys to the adult females and fourth-instar larvae of the mosquitoes of Iran (Diptera: Culicidae). *Zootaxa*. 2009;2078(1):1-33. doi: [10.11646/zootaxa.2078.1.1](https://doi.org/10.11646/zootaxa.2078.1.1).
- Ebrahimzadeh A, Fouladi B, Fazaeli A. High rate of detection of mixed infections of Plasmodium vivax and Plasmodium falciparum in South-East of Iran, using nested PCR. *Parasitol Int*. 2007;56(1):61-4. doi: [10.1016/j.parint.2006.12.001](https://doi.org/10.1016/j.parint.2006.12.001). [PubMed: [17257891](https://pubmed.ncbi.nlm.nih.gov/17257891/)].
- Mirahmadi H, Shahrakipour A, Mehravaran A, Khorashad AS, Rahmati-Balaghaleh M, Zarean M. Evaluation of malaria multiplex/nested PCR performance at low parasite densities and mixed infection in Iran: A country close to malaria elimination. *Infect Genet Evol*. 2018;65:283-7. doi: [10.1016/j.meegid.2018.08.005](https://doi.org/10.1016/j.meegid.2018.08.005). [PubMed: [30096462](https://pubmed.ncbi.nlm.nih.gov/30096462/)].
- Azizi K, Poudat A, Soltani A, Mehranzadeh M. [Fauna and some biological characteristics of anopheles mosquitoes (diptera: Culicidae) in malaria high risk regions: Hormozgan province, 2007-2008]. *Hormozgan medical journal*. 2012;16(4):272-83. Persian.
- Kalantari M, Soltani Z, Ebrahimi M, Yousefi M, Amin M, Shafiei A, et al. [Monitoring of Plasmodium infection in humans and potential vectors of malaria in a newly emerged focus in southern Iran]. *Pathog Glob Health*. 2017;111(1):49-55. Persian. doi: [10.1080/20477724.2016.1271094](https://doi.org/10.1080/20477724.2016.1271094). [PubMed: [28078947](https://pubmed.ncbi.nlm.nih.gov/28078947/)]. [PubMed Central: [PMC5375612](https://pubmed.ncbi.nlm.nih.gov/PMC5375612/)].
- Manouchehri AV, Zaim M, Emadi AM. A review of malaria in Iran, 1975-90. *J Am Mosq Control Assoc*. 1992;8(4):381-5. [PubMed: [1474383](https://pubmed.ncbi.nlm.nih.gov/1474383/)].
- Mirahmadi H, Fallahi S. [Determination of the prevalence and type of plasmodium in mosquitoes present in the city of Nik Shahr, Southern Sistan and Baluchestan Province, IR Iran, by Multiplex Nested-PCR]. *scientific magazine yafte(Yafteh)*. 2019;20(4):63-73. Persian.
- Assmar M, Ter Hovanessian A, Naddaf SR, Piazak N, Masomi H. [PCR Detection of malaria parasites in Anopheles stephensi and Anopheles culicifacies mosquitoes collected from southern endemic foci of Iran]. *Journal of school of public health and institute of public health research*. 2005;3(3):19-26. Persian.
- Oshaghi M, Moradi M, Taghilou B. [Specific detection of malaria parasites using nested-pcr in individual mosquitoes and infected bloods in Chabahar and Iranshahr, Iran]. *Hakim Res J*. 2004;7(3). Persian.
- Yu G, Yan G, Zhang N, Zhong D, Wang Y, He Z, et al. The Anopheles community and the role of Anopheles minimus on malaria transmission on the China-Myanmar border. *Parasit Vectors*. 2013;6(1):264. doi: [10.1186/1756-3305-6-264](https://doi.org/10.1186/1756-3305-6-264). [PubMed: [24034528](https://pubmed.ncbi.nlm.nih.gov/24034528/)]. [PubMed Central: [PMC3856509](https://pubmed.ncbi.nlm.nih.gov/PMC3856509/)].

25. Chang KS, Yoo DH, Ju YR, Lee WG, Roh JY, Kim HC, et al. Distribution of malaria vectors and incidence of vivax malaria at Korean army installations near the demilitarized zone, Republic of Korea. *Malar J.* 2016;**15**(1):259. doi: [10.1186/s12936-016-1301-y](https://doi.org/10.1186/s12936-016-1301-y). [PubMed: [27150110](https://pubmed.ncbi.nlm.nih.gov/27150110/)]. [PubMed Central: [PMC4858856](https://pubmed.ncbi.nlm.nih.gov/PMC4858856/)].
26. Poudat A, Ladoni H, Raeisi A. [Possible factors affecting the malaria situation in the city of Bandar Abbas in 1999-2002]. *Journal of Hormozgan University of Medical Sciences.* 2006;**2**:101-10. Persian.
27. Raeisi A, Nikpour F, Ranjbar KM, Faraji L. [The trend of Malaria in IR Iran from 2002 to 2007]. *Hakim Res J.* 2009;**12**(1):35-41. Persian.
28. Raeisi A, Nejati J, Ansari-moghaddam A, Sakeni M, Faraji L, Paktinat B, et al. Effects of foreign immigrants on malaria situation in cleared up and potential foci in one of the highest malaria burden district of southern Iran. *Malar J.* 2012;**11**(S1). doi: [10.1186/1475-2875-11-s1-p81](https://doi.org/10.1186/1475-2875-11-s1-p81).
29. Vatandoost H, Oshaghi MA, Abaie MR, Shahi M, Yaaghoobi F, Baghahi M, et al. [Bionomics of Anopheles stephensi Liston in the malarious area of Hormozgan province, southern Iran, 2002]. *Acta Trop.* 2006;**97**(2):196-203. Persian. doi: [10.1016/j.actatropica.2005.11.002](https://doi.org/10.1016/j.actatropica.2005.11.002). [PubMed: [16329986](https://pubmed.ncbi.nlm.nih.gov/16329986/)].
30. Nankabirwa JI, Yeka A, Arinaitwe E, Kigozi R, Drakeley C, Kanya MR, et al. Estimating malaria parasite prevalence from community surveys in Uganda: a comparison of microscopy, rapid diagnostic tests and polymerase chain reaction. *Malar J.* 2015;**14**:528. doi: [10.1186/s12936-015-1056-x](https://doi.org/10.1186/s12936-015-1056-x). [PubMed: [26714465](https://pubmed.ncbi.nlm.nih.gov/26714465/)]. [PubMed Central: [PMC4696244](https://pubmed.ncbi.nlm.nih.gov/PMC4696244/)].
31. Gonzalez C, Molina AG, Leon C, Salcedo N, Rondon S, Paz A, et al. Entomological characterization of malaria in northern Colombia through vector and parasite species identification, and analyses of spatial distribution and infection rates. *Malar J.* 2017;**16**(1):431. doi: [10.1186/s12936-017-2076-5](https://doi.org/10.1186/s12936-017-2076-5). [PubMed: [29078770](https://pubmed.ncbi.nlm.nih.gov/29078770/)]. [PubMed Central: [PMC5658941](https://pubmed.ncbi.nlm.nih.gov/PMC5658941/)].
32. Nepomichene TN, Tata E, Boyer S. Malaria case in Madagascar, probable implication of a new vector, Anopheles coustani. *Malar J.* 2015;**14**:475. doi: [10.1186/s12936-015-1004-9](https://doi.org/10.1186/s12936-015-1004-9). [PubMed: [26620552](https://pubmed.ncbi.nlm.nih.gov/26620552/)]. [PubMed Central: [PMC4666205](https://pubmed.ncbi.nlm.nih.gov/PMC4666205/)].
33. Kumar A, Hosmani R, Jadhav S, de Sousa T, Mohanty A, Naik M, et al. Anopheles subpictus carry human malaria parasites in an urban area of Western India and may facilitate perennial malaria transmission. *Malar J.* 2016;**15**:124. doi: [10.1186/s12936-016-1177-x](https://doi.org/10.1186/s12936-016-1177-x). [PubMed: [26919828](https://pubmed.ncbi.nlm.nih.gov/26919828/)]. [PubMed Central: [PMC4769513](https://pubmed.ncbi.nlm.nih.gov/PMC4769513/)].
34. Chinh VD, Masuda G, Hung VV, Takagi H, Kawai S, Annoura T, et al. Prevalence of human and non-human primate Plasmodium parasites in anopheline mosquitoes: a cross-sectional epidemiological study in Southern Vietnam. *Trop Med Health.* 2019;**47**:9. doi: [10.1186/s41182-019-0139-8](https://doi.org/10.1186/s41182-019-0139-8). [PubMed: [30692869](https://pubmed.ncbi.nlm.nih.gov/30692869/)]. [PubMed Central: [PMC6343293](https://pubmed.ncbi.nlm.nih.gov/PMC6343293/)].
35. Ebrahimzadeh A, Mirahmadi H, Mehravaran A, Salimi Khorashad A, Turki H; Dalir. The incidence of current infection with different human malaria species by polymerase chain reaction for diagnosis of suspicious malaria patients on elimination region Sistan and Baluchistan province, Southeast of Iran. *Jundishapur J Microbiol.* 2017;**10**(10). doi: [10.5812/jjm.58254](https://doi.org/10.5812/jjm.58254).
36. Mojahedi AR, Basseri HR, Raeisi A, Pakari A. [Bioecological characteristics of malaria vectors in different geographical areas of Bandar-Abbas county, 2014]. *Int J Prev Med.* 2016;**3**(1):27-18. Persian.
37. Pemola Devi N, Jauhari RK. Relationship between Anopheles fluviatilis & A. stephensi (Diptera: Culicidae) catches & the prevalence of malaria cases at Kalsi area in Dehradun district (Uttaranchal). *Indian J Med Res.* 2006;**123**(2):151-8. [PubMed: [16575114](https://pubmed.ncbi.nlm.nih.gov/16575114/)].
38. Qureshi NA, Fatima H, Afzal M, Khattak AA, Nawaz MA. Occurrence and seasonal variation of human Plasmodium infection in Punjab Province, Pakistan. *BMC Infect Dis.* 2019;**19**(935):1-13. doi: [10.1186/s12879-019-4590-2](https://doi.org/10.1186/s12879-019-4590-2). [PubMed: [31694574](https://pubmed.ncbi.nlm.nih.gov/31694574/)]. [PubMed Central: [PMC6836532](https://pubmed.ncbi.nlm.nih.gov/PMC6836532/)].