Arch Clin Infect Dis. 2021 June; 16(3):e103728. doi: 10.5812/archcid.103728.

Published online 2021 July 7. **Research Article**

A Comprehensive Survey of Asymptomatic Malaria Cases in an Endemic Focus in Iran: A Successful Experience on the Road to Eliminate Malaria

Habibollah Turki [1], Mohammad Shekari [2] and Aboozar Soltani [3, *]

[1] Molecular Medicine Research Center, Hormozgan Health Institute, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[2] Department of Genetics, Faculty of Medicine, Hormozgan University of Medical Sciences, Bandar Abbas, Iran
[3] Department of Medical Entomology and Vector Control, Research Center for Health Sciences, Institute of Health, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran

[*] *Corresponding author*: Department of Medical Entomology and Vector Control, Research Center for Health Sciences, Institute of Health, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran. Email: abu2sol@yahoo.com

Received 2020 April 14; **Revised** 2021 May 05; **Accepted** 2021 May 09.

Abstract

Background: Malaria is one of the important infectious blood diseases caused by the protozoan parasite of the genus *Plasmodium* and transmitted by female *Anopheles* mosquito bites. A malaria elimination plan is currently being followed in Hormozgan Province. The robust malaria surveillance system with appropriate active case findings, especially asymptomatic cases, plays an important role in the malaria elimination program.
Objectives: The main objectives of this research were to determine the presence and prevalence of asymptomatic malaria cases and monitor asymptomatic parasitic reservoirs in Jask District, Hormozgan Province.
Methods: This cross-sectional study aimed to evaluate and monitor asymptomatic cases in the Jask District. The purpose and stages of the study were explained to all participants/parents, and written informed consent was obtained. A total of 230 asymptomatic residents (124 females and 86 males) were randomly selected, and their blood samples (3 mL) were taken to assess *Plasmodium* infection using microscopic, RDT, and molecular (18ssrRNA) methods.
Results: Of the 230 studied cases, 54.8% were females, and 454.2% were males. The age range was four to 65 years old, and the mean age was 24.5. None of the diagnostic methods, including the microscopic, serological, and molecular techniques, could find asymptomatic malaria cases in the study area.
Conclusions: It can be concluded that Malaria Elimination Program is feasible in the Jask Region irrespective of asymptomatic parasitic reservoirs. The results also emphasize a robust and efficient malaria surveillance system to diagnose and treat positive cases and monitor treated cases successfully. Ongoing and continuous studies are recommended in the high-risk malarious area of Hormozgan Province to monitor asymptomatic cases of malaria.

Keywords: Malaria, *Plasmodium*, Surveillance System, Elimination Program

1. Background

Malaria is one of the important infectious blood diseases caused by the protozoan parasite of the genus *Plasmodium* and transmitted by female *Anopheles* mosquito bites (1, 2). To date, five species of *Plasmodium* have been reported to cause human malaria, among which *Plasmodium falciparum* is considered a malignant species and linked to the highest rate of deaths from malaria. Another species is *Plasmodium vivax* accounting for the most positive cases of malaria in the world. However, this species is associated with a lower incidence of malaria deaths. Two species of *Plasmodium ovale* and *Plasmodium malariae* generally have been reported to cause a moderate form of the disease. The last species is *Plasmodium knowlesi* that has been responsible for sporadic cases of malaria in southeast Asia in recent years (3).

Globally, 229 million cases of malaria have been reported by the World Health Organization (WHO) in 2020 of which, 409,000 died. Also, more than 87 countries have reported malaria transmission (4). The impact of malaria disease on public health and health promotion criteria located malaria in the focus of international organizations related to the health of societies. These organizations approved the conclusive programs of control, elimination, and eradication of malaria (5).

In recent years, there has been a significant decline in the number of positive cases of malaria in Iran. According to WHO, Iran is in the process of eliminating malaria. The first phase of the malaria elimination program (2010 - 2015), with the support of WHO, was successfully implemented in Iran, and its second phase (2016 - 2020) has been started since 2016 and is running now (3). Given that no local transmission of malaria was observed in Iran during 2018 - 2019 according to the WHO report, and considering the appropriate status of the malaria elimination plan, obtaining a certificate of malaria elimination is now possible (4, 6).

Asymptomatic malaria is a major challenge to the malaria elimination plan. It causes a reservoir, which can contribute to the ongoing transmission of disease (7). The reservoir of asymptomatic malaria has been investigated in some studies in Iran (8-16) and other countries (17-25). The results reported in these studies are inconsistent in terms of the presence of positive cases. A malaria elimination plan is currently being followed in Hormozgan Province. The robust malaria surveillance system with appropriate active case findings can play an important role in the malaria elimination program (26).

2. Objectives

The main objectives of this research were to determine the presence and prevalence of asymptomatic malaria cases and monitor asymptomatic parasitic reservoirs in Jask District, Hormozgan Province. According to the results of previous studies showing that routine diagnostic techniques (microscopy and RDT) do not make enough sensitivity to diagnose asymptomatic malaria cases, a molecular assay as a robust and sensitive method was added in this study. The findings of the present study could support the successful implementation of the malaria elimination program in Hormozgan and Iran by providing basic information and identifying challenges.

3. Methods

3.1. Study Area

This study was performed in Jask County as an endemic area of malaria in Hormozgan Province. Jask County with an area of 16136.2 km^2, located at a longitude of 57°10' - 59°16'E and latitude of 25°23' - 26°13'N, is in the southeast of Hormozgan Province. Jask County is bounded on the east and northeast by Sistan and Baluchestan Province. The south and southwest of the county are adjacent to the waters of the Oman Sea and the Strait of Hormuz (Figure 1) (http://hormozgan.rmto.ir/English/Pages/Introduction.aspx). According to the 2017 census, the population of Jask County was about 58,884. The city's climate is described as hot and dry in summer and temperate in winter, with a relative humidity of more than 50% across the coastal areas (Figure 1). Jask County is one of the malarious areas of Hormozgan Province in which malaria has been reported in recent years. Due to proximity to Sistan and Baluchistan Province, the arrival of foreigners, population displacement, and appropriate weather conditions, Jask County is susceptible to malaria transmission almost year-round. According to the WHO benchmark, Jask is now in the malaria elimination phase (API < 1/1000 per year).

3.2. Study Population and Inclusion Criteria

This cross-sectional study aimed to evaluate and monitor asymptomatic cases in the Jask District. A total of 230 (asymptomatic residents 124 females and 86 males) were randomly selected, and their blood samples (3 mL) were taken to assess *Plasmodium* infection using microscopic, RDT, and molecular (18ssrRNA) methods (Table 1).

The purpose and stages of the study were explained to all participants/parents, and written informed consent was obtained. After interviewing and recording the demographic data (name, age, sex, job, immigration history, and malaria history), the presence or absence of clinical malaria signs was evaluated. The current study included those participants who had no clinical symptoms of malaria, and excluded those who had used anti-malarial drugs in the past month and traveled to other malaria-endemic areas in the past three months. Pregnant women and people under four or over 60 years old were also excluded from the study.

3.2.1. Ethical Approval

The project was done as per the ethical principles and the national norms and standards for conducting medical research in Iran. The study was approved by the Iran National Committee for Ethics in Biomedical Research (approval ID: IR.HUMS.REC.92-4-1).

3.3. Diagnostic Tests

3.3.1. Microscopic Examination (Direct Examination Method)

The microscopic or direct diagnosis method of malaria was carried out according to the guidelines approved by the WHO (27). The microscopic technique is the common and gold standard procedure of detecting malaria parasites in suspicious specimens. Blood samples were taken from the enrolled participants. Thin and thick blood smears were prepared. After making the blood smears on slides, only thin smears were fixed with methanol. Thin and thick blood films were stained with the Giemsa

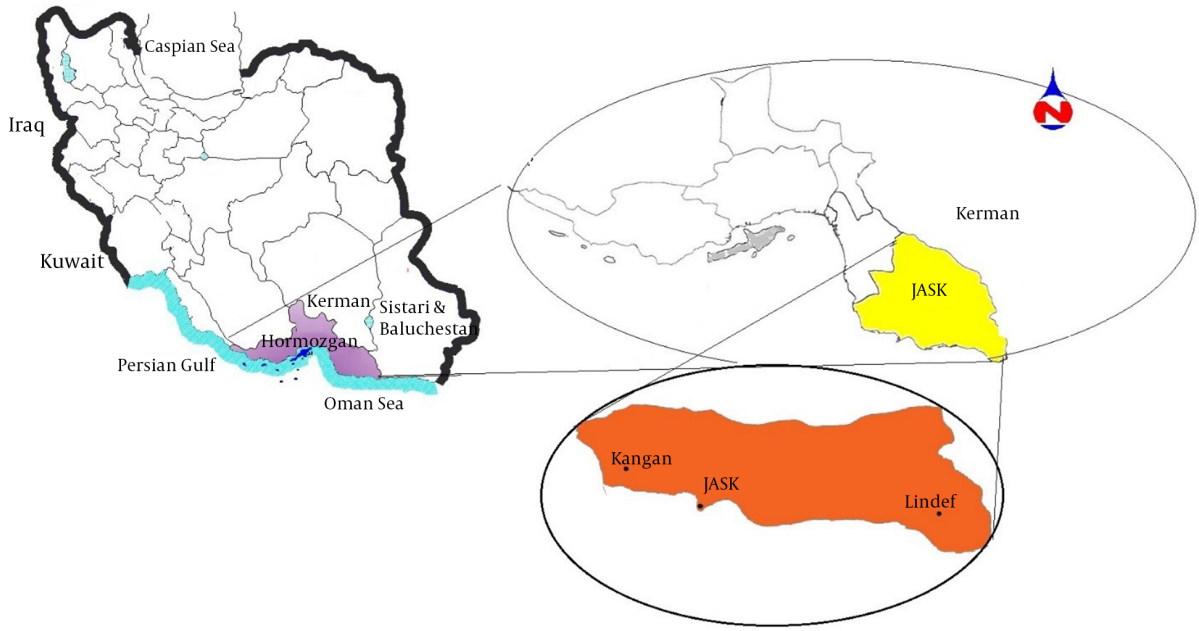

Figure 1. Iran's map indicating the study area (Jask District, Hormozgan Province)

Table 1. Distribution of Samples Based on Age and Sex in the Endemic Area of Jask District

Study Subjects	Age Group, y				Gender	
Group	**< 15**	**15 - 30**	**30 - 45**	**> 45**	**Female**	**Male**
Subject, No. (%)	29 (12.6)	154 (67)	37 (16)	10 (4.4)	126 (54.8)	104 (45.2)
Total	230 (100)					

method conveniently. The microscopic examination of stained slides was performed via Immersion oil at 1000× magnification (28). The microscopic evaluation of all participants was performed by two different groups (double-blind) to identify asymptomatic patients and individuals with low parasitemia.

3.3.2. Rapid Diagnostic Tests (RDTs)

The rapid malaria detection test is a diagnostic method used in association with the microscopic test to identify malaria, based on the formation of antigen/antibody complex. This prompt and accurate method relies on the immunochromatography mechanism.

All samples were tested with the First Response® Malaria Combo (pLDH/HRP2) card test (Premier Medical Corporation Ltd., Mumbai, India). According to the manufacturer's instructions, five microliters of blood samples of the patient's finger-prick were poured into the RDTs kit well. In the next step, three drops of buffer were added to the buffer well located on the kit to lyse RBCs. After 20 minutes, the results were analyzed according to lines that were visible in the test and control area (29).

3.3.3. Molecular Technique (Nested PCR)

As described by Snounou et al. (30), the molecular method of Nested PCR was implemented for the recognition of malaria parasites in samples based on the protocol.

The parasite DNA was extracted from specimens by the Genomic DNA Blood/Culture Cell Mini kit of "Yekta Tajhiz Azma" Iran Company. The Nested PCR technique used two sets of primers and was performed in two successive reactions, including genus identification and species identification of *P. vivax* and *P. falciparum* in taken samples of the study. In the first step of amplification (Nested PCR-1), 2 μL of extracted DNA of each sample was added to other reaction items. The reaction continued by a specific primer for *Plasmodium* (1200 bp). The reaction was done in the final volume of 50 μL, and the PCR was performed on the appropriate program by a thermocycler.

Nested PCR-2 involved the use of first-stage amplified

product as a DNA template for the next reaction, and the species-specific primer was used to identify *P. vivax* (120 bp) and *P. falciparum* (205 bp). Each step was repeated for 25 - 30 cycles, and the annealing temperature was optimized at 72°C for both steps. Finally, to analyze the Nested PCR-2 products, electrophoresis was conducted in the presence of appropriate standard 100 bp molecular markers, and the gel photos were recorded by digital imaging for the final report (31). Positive and negative controls were used in each reaction series along with PCR running of the samples. The DNA extracted from the blood of healthy individuals who had not previously had malaria and had no history of traveling to endemic regions was applied as negative controls. For positive control preparation, the parasite DNA was extracted from blood samples of patients whose malaria had been confirmed by microscopic examination.

4. Results

Table 1 shows the demographic characteristics of the participants in this research. Of the 230 studied cases, 54.8% were females, and 454.2% were males. The age range was four to 65 years old, and the mean age was 24.5 years old. No positive cases of malaria parasites were found using microscopic, RDT, and molecular methods. It should be noted that the microscopic experiments were performed by double-blind microscopists (Table 2). In the molecular approach, positive and negative controls were used in each series of experiments (Figure 2). In summary, these results indicate that there were no asymptomatic malaria cases in Jask County despite using microscopic, serological, and molecular methods (Table 2).

5. Discussion

The elimination of malaria is one of the common goals of the Iranian health system and the World Health Organization. Active malaria case finding and detection of asymptomatic parasitic reservoirs are the most important strategies in the malaria elimination program (32). Despite using the molecular sensitive method for diagnosis, the results of this study showed that the asymptomatic parasitic reservoir was not observed in Jask. The absence of an asymptomatic parasitic reservoir indicates the existence of a robust surveillance system to diagnose and treat positive cases and monitor them.

To successfully apply the malaria elimination program, it is necessary to detect and monitor asymptomatic cases. Being a major challenge to the malaria elimination plan, asymptomatic malaria causes a reservoir, contributing to the ongoing local transmission of the disease. In this study, the PCR technique was used in addition to routine diagnostic methods (microscopic and RDT) because of the high sensitivity of the molecular method.

The findings of this study are in line with previous studies conducted in some parts of Iran, including Bashagard (15), Kerman (15, 16), Rudan (13), and Iranshahr (9). Despite using a sensitive molecular method for diagnosis, there have been no reports of asymptomatic cases in some of these cities. However, positive cases of asymptomatic malaria have been reported in cities of Minab (12), Bashagard (11), Iranshahr (8), and Bandar-Abbas (10). The most important reason to explain the disagreement between this study and previous studies may be the robust malaria surveillance system in the area (Jask).

Recently, a cross-sectional study was conducted on the situation of asymptomatic malaria among Iranian native and Afghan and Pakistani immigrants in the southeastern province of Sistan and Baluchistan. A total of 271 individuals were surveyed based on the microscopic method, Rapid Diagnostic test (RDT), and PCR techniques. Similar to our results, none of the examined samples was diagnosed as malaria-positive cases (33). Furthermore, there are similarities between the results reported in this study and those described by other researchers in Honduras (21), Sri Lanka (20), and India (19), which have not reported positive malaria cases despite the use of sensitive and robust molecular methods.

However, our findings are contrary to previous studies conducted in Bhutan (24), India (23), Thailand (18), China-Myanmar border (25), Ethiopia (22), and Brazil (17), which found asymptomatic malaria cases. A similar study was conducted in Ghana in 2018. Asymptomatic adult residents from five villages were screened for *Plasmodium* species using RDT and molecular techniques. Quite contrary to our results, the molecular prevalence of asymptomatic *Plasmodium* infection was 73%, and a 32% infection rate was detected by RDT (34). This discrepancy can be largely attributed to differences in the epidemiological characteristics and status of malaria transmission in the studied areas. Other possible explanations for this dissimilarity are differences in the genetic characteristics of the parasites and hosts, species diversity and vectorial capacity of vectors, and displacement or relocation of human populations (35).

The key strengths of this study are the simultaneous use of molecular sensitive techniques along with routine methods of malaria detection, as well as a wide field selected for sampling in the endemic area of Jask. The main limitations of this research include the small sample size and the impossibility to monitor the cases due to the cross-sectional design of the study. Despite the use of a molecular sensitive technique and routine methods of malaria

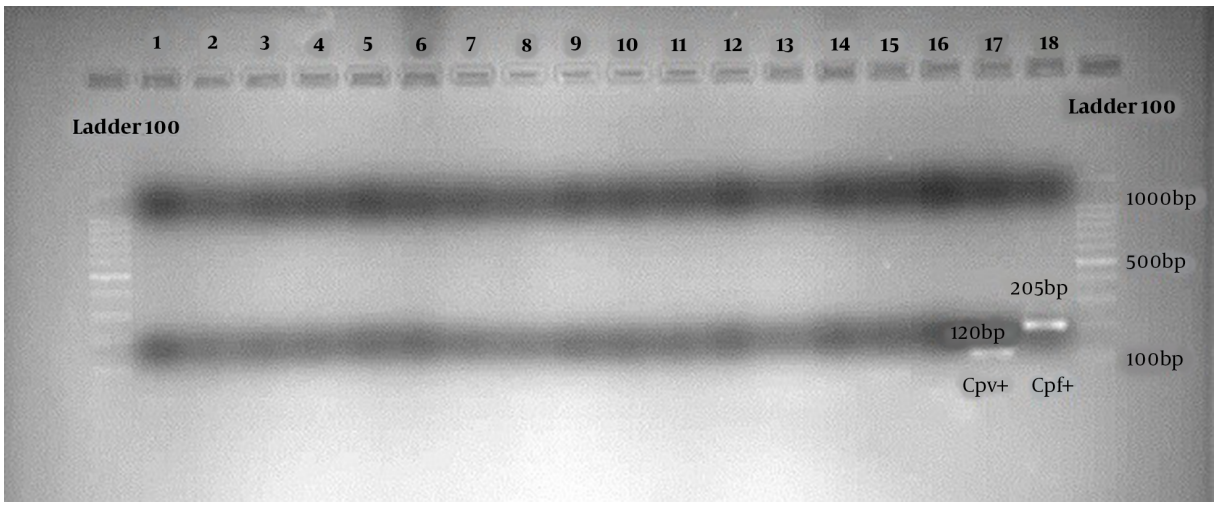

Figure 2. The results of PCR product on samples stained with electrophoresis gel; molecular marker (100 bp Cinnagen, Iran) (the first and last lanes), positive control of *Plasmodium vivax* (lane 17), positive control of *Plasmodium falciparum* (lane 18), negative samples (lanes 1-16)

Table 2. Descriptive Statistics of Asymptomatic Cases of Malaria in the Endemic Area of Jask District According to Examination Tests

Status	Method					
	Microscopic		RDT		Molecular	
	Positive	Negative	Positive	Negative	Positive	Negative
Subject, No. (%)	0 (0)	230 (100)	0 (0)	230 (100)	0 (0)	230 (100)
Total	230 (100)					

detection, the asymptomatic parasitic reservoirs in the malaria-endemic area of Jask were not reported, indicating a successful implementation of the malaria elimination program in this area without worrying about low parasitemia and asymptomatic malaria cases.

According to the world malaria report 2020, local malaria transmission was not observed in Iran in 2018 and 2019. Due to the lack of local malaria transmission in 2020 and the completion of a three-year period for no local malaria transmission, conditions for obtaining a Malaria Eliminate Certificate have been provided by the WHO in Iran (4). Along with other similar research, the results of our study, as valid evidence, will facilitate the process of obtaining the certification of malaria-free for the country. In addition, the findings of this research contribute in many ways to identify the challenges of the malaria elimination program.

5.1 Conclusions

It can be concluded that Malaria Elimination Program is feasible in the Jask Region irrespective of asymptomatic parasitic reservoirs. The results also emphasize a robust and efficient malaria surveillance system to diagnose and treat the positive cases and monitor the treated cases successfully. Ongoing and continuous studies are recommended in the high-risk malarious area of Hormozgan province to monitor asymptomatic cases of malaria.

Footnotes

Authors' Contribution: HT designed and conceptualized the study. HT, MS, and AS conducted the parasitological and molecular surveys and analyzed data. HT and AS drafted the manuscript. All the authors participated in writing the manuscript. All the authors have read and approved the final manuscript.

Conflict of Interests: No conflict of interest is declared.

Ethical Approval: The study was approved by the Iran National Committee for Ethics in Biomedical Research (approval ID: IR.HUMS.REC.92-4-1).

Funding/Support: It was not declared by the authors.

Informed Consent: The purpose and stages of the study were explained to all participants/parents, and written informed consent was obtained.

References

1. Fornace KM, Diaz AV, Lines J, Drakeley CJ. Achieving global malaria eradication in changing landscapes. *Malar J*. 2021;**20**(1):69. doi: 10.1186/s12936-021-03599-0. [PubMed: 33530995]. [PubMed Central: PMC7856737].
2. Keshavarzi D, Soltani Z, Ebrahimi M, Soltani A, Nutifafa GG, Soltani F, et al. Monthly prevalence and diversity of mosquitoes (Diptera: Culicidae) in Fars Province, Southern Iran. *Asian Pac J Trop Dis*. 2017;**7**(2):112–20. doi: 10.12980/apjtd.7.2017D6-369.
3. Schapira A, Zaim M, Raeisi A, Ranjbar M, Kolifarhood G, Nikpour F, et al. *History of the successful struggle against malaria in the Islamic Republic of Iran*. Tehran: Neekpey; 2018. p. 27–8.
4. World Health Organization. *World malaria report 2020: 20 years of global progress and challenges*. 2020.
5. Tanner M, Greenwood B, Whitty CJ, Ansah EK, Price RN, Dondorp AM, et al. Malaria eradication and elimination: views on how to translate a vision into reality. *BMC Med*. 2015;**13**:167. doi: 10.1186/s12916-015-0384-6. [PubMed: 26208740]. [PubMed Central: PMC4514994].
6. World Health Organization. *World malaria report 2019*. 2019.
7. Jaiteh F, Okebe J, Masunaga Y, D'Alessandro U, Achan J, Gryseels C, et al. Understanding adherence to reactive treatment of asymptomatic malaria infections in The Gambia. *Sci Rep*. 2021;**11**(1):1746. doi: 10.1038/s41598-021-81468-1. [PubMed: 33462329]. [PubMed Central: PMC7813830].
8. Nateghpour M, Akbarzadeh K, Farivar L, Amiri A. Detection of asymptomatic malaria infection among the Afghani immigrant population in Iranshahr district of southeastern Iran. *Bull Soc Pathol Exot*. 2011;**104**(4):321–3. doi: 10.1007/s13149-011-0134-8. [PubMed: 21312079].
9. Pirahmadi S, Zakeri S, Raeisi A. Absence of Asymptomatic Malaria Infection in a Cross-sectional Study in Iranshahr District, Iran under Elimination Programmes. *Iran J Parasitol*. 2017;**12**(1):90–100. [PubMed: 28761465]. [PubMed Central: PMC5522703].
10. Rashid G, Soltani A, Eftekhar E, Shafiei R, Turki H. Immigrants: Big Challenge and Silent Threat to Implement Malaria Elimination Program in Hormozgan Province, Iran. *Jundishapur J Microbiol*. 2020;**13**(1). doi: 10.5812/jjm.99725.
11. Shahbazi A, Farhadi P, Yerian M, Bazmani A, Nakhjiri SK, Rasouli A, et al. Detection of Asymptomatic Carriers of Plasmodium vivax among Treated Patients by Nested PCR Method in Minab, Rudan and Bashagard, Iran. *Iran J Parasitol*. 2013;**8**(4):586–92. [PubMed: 25516740]. [PubMed Central: PMC4266123].
12. Turki H, Raeisi A, Malekzadeh K, Ghanbarnejad A, Zoghi S, Yeryan M, et al. Efficiency of Nested-PCR in Detecting Asymptomatic Cases toward Malaria Elimination Program in an Endemic Area of Iran. *Iran J Parasitol*. 2015;**10**(1):39–45. [PubMed: 25904944]. [PubMed Central: PMC4403538].
13. Turki H, Rashid G, Shekari M, Raeisi A, Sharifi-Sarasiabi K. Malaria Elimination Program: Absence of asymptomatic malaria and low parasitic in endemic area of Rudan district, Hormozgan Province, Iran. *Hormozgan Med J*. 2017;**21**(4):225–31. doi: 10.29252/hmj.21.4.225.
14. Turki H, Soltani A. Follow-Up and Monitoring of Malaria Treated Cases Toward Malaria Elimination Program in Bashagard District, Hormozgan Province, Iran, in 2016. *Jundishapur J Microbiol*. 2019;**In Press**(In Press). doi: 10.5812/jjm.85267.
15. Turki H, Zoghi S, Mehrizi AA, Zakeri S, Raeisi A, Khazan H, et al. Absence of asymptomatic malaria infection in endemic area of bashagard district, hormozgan province, iran. *Iran J Parasitol*. 2012;**7**(1):36–44. [PubMed: 23133470]. [PubMed Central: PMC3488819].
16. Zoghi S, Mehrizi AA, Raeisi A, Haghdoost AA, Turki H, Safari R, et al. Survey for asymptomatic malaria cases in low transmission settings of Iran under elimination programme. *Malar J*. 2012;**11**:126. doi: 10.1186/1475-2875-11-126. [PubMed: 22533733]. [PubMed Central: PMC3464154].
17. Almeida ACG, Kuehn A, Castro AJM, Vitor-Silva S, Figueiredo EFG, Brasil LW, et al. High proportions of asymptomatic and submicroscopic Plasmodium vivax infections in a peri-urban area of low transmission in the Brazilian Amazon. *Parasit Vectors*. 2018;**11**(1):194. doi: 10.1186/s13071-018-2787-7. [PubMed: 29558985]. [PubMed Central: PMC5859403].
18. Baum E, Sattabongkot J, Sirichaisinthop J, Kiattibutr K, Davies DH, Jain A, et al. Submicroscopic and asymptomatic Plasmodium falciparum and Plasmodium vivax infections are common in western Thailand - molecular and serological evidence. *Malar J*. 2015;**14**:95. doi: 10.1186/s12936-015-0611-9. [PubMed: 25849211]. [PubMed Central: PMC4342942].
19. Dhiman S, Goswami D, Rabha B, Yadav K, Chattopadhyay P, Veer V. Absence of asymptomatic malaria in a cohort of 133 individuals in a malaria endemic area of Assam, India. *BMC Public Health*. 2015;**15**:919. doi: 10.1186/s12889-015-2294-0. [PubMed: 26384971]. [PubMed Central: PMC4575429].
20. Fernando SD, Abeyasinghe RR, Galappaththy GN, Rajapaksa LC. Absence of asymptomatic malaria infections in previously high endemic areas of Sri Lanka. *Am J Trop Med Hyg*. 2009;**81**(5):763–7. doi: 10.4269/ajtmh.2009.09-0042. [PubMed: 19861607].
21. Pinto A, Ávalos S, Girón C, Mejía RE, Fontecha G. Absence of asymptomatic malaria in pregnant women of Honduras. *Revista Panamericana de Enfermedades Infecciosas*. 2018:68–72.
22. Tadesse FG, van den Hoogen L, Lanke K, Schildkraut J, Tetteh K, Aseffa A, et al. The shape of the iceberg: quantification of submicroscopic Plasmodium falciparum and Plasmodium vivax parasitaemia and gametocytaemia in five low endemic settings in Ethiopia. *Malar J*. 2017;**16**(1):99. doi: 10.1186/s12936-017-1749-4. [PubMed: 28253867]. [PubMed Central: PMC5335517].
23. van Eijk AM, Sutton PL, Ramanathapuram L, Sullivan SA, Kanagaraj D, Priya GSL, et al. The burden of submicroscopic and asymptomatic malaria in India revealed from epidemiology studies at three varied transmission sites in India. *Sci Rep*. 2019;**9**(1):17095. doi: 10.1038/s41598-019-53386-w. [PubMed: 31745160]. [PubMed Central: PMC6863831].
24. Wangchuk S, Gyeltshen S, Dorji K, Wangdi T, Dukpa T, Namgay R, et al. Malaria elimination in Bhutan: asymptomatic malaria cases in the Bhutanese population living in malaria-risk areas and in migrant workers from India. *Rev Inst Med Trop Sao Paulo*. 2019;**61**. e52. doi: 10.1590/S1678-9946201961052. [PubMed: 31531630]. [PubMed Central: PMC6746194].
25. Zhao Y, Zeng J, Zhao Y, Liu Q, He Y, Zhang J, et al. Risk factors for asymptomatic malaria infections from seasonal cross-sectional surveys along the China-Myanmar border. *Malar J*. 2018;**17**(1):247. doi: 10.1186/s12936-018-2398-y. [PubMed: 29973194]. [PubMed Central: PMC6032786].
26. World Health Organization. *Malaria surveillance, monitoring and evaluation: a reference manual*. 2018.
27. World Health Organization. *Malaria microscopy quality assurance manual-version 2*. 2016.
28. Nateghpour M, Tourki H, Keshavarz H, EDRISIAN GH, Mohebali M, Foroushani AR. A parasitological and serological study in malaria suspected patients in Hormozgan province, southeastern Iran. *Iran Red Crescent Med J*. 2010;**12**(3):242–6.
29. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. *Clin Microbiol Infect*. 2013;**19**(5):399–407. doi: 10.1111/1469-0691.12151. [PubMed: 23438048].
30. Snounou G, Viriyakosol S, Jarra W, Thaithong S, Brown KN. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. *Mol Biochem Parasitol*. 1993;**58**(2):283–92. doi: 10.1016/0166-6851(93)90050-8. [PubMed: 8479452].
31. Ebrahimzadeh A, Nouri Dalir S, Mirahmadi H, Mehravaran A, Salimi Khorashad A, Turki H. 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.
32. Zare M, Farshidi H, Soleimani-Ahmadi M, Jaberhashemi SA, Sanei-Dehkordi A. Significant decline of malaria incidence in a low socioeconomic area in the southeast of Iran: 10 years field assessment during malaria elimination programme. *J Parasit Dis*. 2021:1–9. doi: 10.1007/s12639-021-01391-0.
33. Askari S, Nateghpour M, Motevalli Haghi A, Farivar L, Raeisi A, Mohebali M. Situation of Asymptomatic Malaria among Iranian Native and Afghan and Pakistani Immigrants in a Malarious Area under the National Malaria Elimination Program of Iran. *Iran J Parasitol*. 2020;**15**(4):530–6. doi: 10.18502/ijpa.v15i4.4858. [PubMed: 33884010]. [PubMed Central: PMC8039483].
34. Heinemann M, Phillips RO, Vinnemeier CD, Rolling CC, Tannich E, Rolling T. High prevalence of asymptomatic malaria infections in adults, Ashanti Region, Ghana, 2018. *Malar J*. 2020;**19**(1):366. doi: 10.1186/s12936-020-03441-z. [PubMed: 33046056]. [PubMed Central: PMC7552528].
35. Mirahmadi H, Spotin A, Fallahi S, Taghipour N, Turki H, Seyyed Tabaei SJ. Cloning and Sequence Analysis of Recombinant Plasmodium vivax Merozoite Surface Protein 1 (PvMSP-142 kDa) In pTZ57R/T Vector. *Iran J Parasitol*. 2015;**10**(2):197–205. [PubMed: 26246817]. [PubMed Central: PMC4522295].