Original Article

Absence of Asymptomatic Malaria Reservoirs in an Area with a Previous History of Local Malaria Transmission: A Successful Experience in Line with the Malaria Elimination Program in Iran

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Abstract

Background: Asymptomatic malaria is a major challenge to be addressed in the implementation of the malaria elimination program. The main goal of the malaria surveillance system in the elimination phase is to identify reliably all the positive cases of malaria reliably (symptomatic and asymptomatic) in the shortest possible time. This study focused on the monitoring of asymptomatic malaria reservoirs in areas where local transmission had been previously established.
Methods: It was a case-study approach that was conducted in the Anarestan area. A total of 246 residents and immigrants living in the area at the age range of 4–60 years old were randomly selected to be tested for malaria by microscope, RDT, and nested-PCR techniques. The inclusion criterion for participants to be entered into the study was the absence of specific symptoms of malaria. Moreover, participants who have been taking antimalarials for the last month were excluded from the study.
Results: The results indicated no positive cases of asymptomatic malaria among the participants tested by all methods.
Conclusion: The results of this study have shown that, without concerns for asymptomatic parasitic patients, a malaria elimination program has been successfully implemented within the studies area. In addition, the findings emphasized the existence of a strong malaria surveillance system in this area.

Keywords: Asymptomatic malaria; *Plasmodium*; Elimination; Surveillance system

Introduction

Malaria is known as one of the most important tropical diseases that has a special importance in the health programs of the World Health Organization (WHO) and has attracted the attention of researchers in different countries. The disease is caused by the protozoan parasite, the genus of *Plasmodium*, which is transmitted by the bites of female *Anopheles* mosquitoes. Human malaria is caused by five species of *Plasmodium* including *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi* (zoonotic species) (1). *Plasmodium cynomolgi* is rarely the cause of some human malaria cases (2).

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In 2021, WHO estimated that there were approximately 247 million malaria cases in the world, resulting in 619,000 deaths, with most cases and deaths occurring in African countries (3). Malaria can influence some aspects of life including public health, economy, and human societies. This issue has caused this disease to be at the center of interest for international health-related organizations. Eventually, the WHO has planned comprehensive programs to control, eliminate, and eradicate the malaria (4). Finally, in recent years, the malaria elimination program (MEP) has been more closely scrutinized. The number of malaria cases in Iran has decreased significantly in recent years showing the effectiveness of preventative measures (5). This success led Iran to become a candidate for the MEP according to the WHO criteria (5). Therefore, the elimination of malaria became a major issue in Iran's health system. In Iran, MEP has been established since 2010 on the technical support of WHO and is currently being implemented successfully (5).

In recent years, the local transmission of malaria in Iran has been reported to be close to zero. If this reduction trend continues in the coming years, Iran could obtain a malaria elimination certificate according to the WHO benchmarks (3).

Early diagnosis and timely treatment of malaria is one of the most important strategies in implementing the MEP (3).

Implementing a MEP requires robust, effective and efficient malaria surveillance systems that can reliably and in the shortest possible time identify the problem and provide a clear and practical solution. The main goal of the malaria surveillance system in the elimination phase is to identify all the positive cases of malaria (symptomatic and asymptomatic) and ensure complete and timely treatment (Radical treatment) to stop the malaria transmission cycle. Asymptomatic malaria refers to the presence of the malaria parasite in human blood, which is asymptomatic and provides a reservoir for transmission (4, 6). The malaria surveillance system in Iran was designed to eliminate the disease in the country by 1404. The program is based on three important strategies: improving access to malaria diagnosis and treatment services, consolidation the integrated vector management (IVM) and reinforcing the malaria surveillance system. This system also emphasizes on appropriate and timely response to potential outbreaks (5). There is an urgent need to address the problems caused by asymptomatic cases in MEP (6, 7). Some researchers performed studies and emphasis on recognizing malaria parasitic reservoirs in Iran (8–19) and other countries (20–32). The plan to eliminate malaria in Iran is currently being pursued with a focus on areas with a history of transmission. Case finding and the establishment of a strong malaria surveillance system play an important role in the successful implementation of the MEP (33). Reporting even one case of local malaria transmission is a major challenge facing the MEP and hinder obtaining a malaria elimination certificate from the World Health Organization.

The biggest challenge faced by the MEP is to report even one case of local malaria transmission, which hider obtaining a WHO malaria elimination certificate. No- symptoms malaria cases are the big challenges of the MEP. Routine methods for diagnosing symptomatic cases of malaria include microscopic methods (Gold standard) and Rapid Diagnostic Test (RDT). However, in order to diagnose asymptomatic and low parasitemia cases, it is necessary to use a sensitive molecular technique along with routine diagnostic methods.

In terms of ecology and climatic conditions, Anarestan region has the capacity to establish a malaria transmission cycle. After a long period of absence of malaria in this region, in 2016, 12 cases of local transmission of malaria were reported, mainly Plasmodium vivax. This issue drew the attention of the health system to this area, and this study was designed to discover asymptomatic parasitic reservoirs in this area.

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To evaluate MEP, current research was designed to monitor no-symptoms malaria reservoirs and determine the frequency of asymptomatic malaria cases in areas with a previous history of local malaria transmission using routine methods and Nested-PCR technique.

Materials and Methods

Study area

This research was conducted in Anarestan region of Kazerun County in Fars Province. Kazerun is located in the west of Fars Province with geographical coordinates of 51°39' 15"E and 29° 37'10"N. According to the 2016 census it has 96,683 populations and 28,988 households (34). This region has boundary with Shiraz from the east, Noorabad from the north, Bushehr from the west and Frashband from the southeast (Fig. 1).

Kazerun has a diverse climate with a cold region in the north and a hot and dry region in the south. In addition to runoff from raining, traditional irrigation systems cause a lot of larvae breeding places in this area. Proper ecological conditions and the presence of abundant the *Anopheles* larval breeding places have made the Anarestan region potentially high risk in terms of malaria transmission. For this reason, in 2016, local malaria transmission was reported due to the presence of the malaria imported cases. Therefore, the Iranian Center for Malaria Management and Control emphasizes to study and to identify the asymptomatic parasitic reservoirs in this area**.**

Study population and inclusion criteria

A case-study approach was conducted in Anarestan to detect asymptomatic malaria cases and assess the efficacy of the MEP in this region. The study population included native people and immigrants living in this area. A total of 246 samples were randomly selected according to the Cochran's formula (35). A comprehensive explanation of the plan was given to the participants and the form of ethical consent was completed by them. The demographic information of individuals, including name and surname, age, gender, nationality, history of malaria infection, and history of travel to the endemic region of malaria were recorded using questionnaire (Table 1).

This research has been approved with ethics code of HUMS.REC.1396.155 in Hormozgan University of Medical Sciences. The study population included local residents and immigrants living in the area who did not have specific symptoms of malaria (chill, fever, sweat). Participants who had been using anti-malarial drugs for the past month were excluded from the study. In terms of age, participants who were 4–60 years old entered into the study.

Diagnostic tests

Two ml of blood was taken from each participant for molecular examination. For the microscopic diagnosis of malaria, thin and thick blood smears were prepared and a simultaneous RDT was performed for all participants.

Microscopic examination

The gold standard and the basic laboratory diagnostic method for malaria is a microscopic peripheral blood smear, which requires good quality blood samples to be accurately diagnosed.

Microscopy method was conducted based on guidelines approved by (WHO) (34). In brief, a sterile lancet used to scratch the tip of the participants' finger. Then, thin and thick blood smears were prepared and thin smears were fixed using methanol. Finally, the blood smear was stained with the Giemsa method and examined using immersion oil microscope (OLYMPUS-CX21-x1000) to detect malaria parasites (36, 37). To assess the accuracy of the microscopic results, the peripheral blood smear of all participants was re-examined blinded by expert microscopists.

Rapid Diagnostic Tests (RDTs)

In recent years, simple and fast malaria detection methods have been used along with microscopic techniques in the malaria control and elimination program. RDTs can detect parasite antigens including *P. falciparum* histidine rich protein 2 (PfHRP-2), parasite lactate dehydrogenase (pLDH), aldolase and glutamate dehydrogenase (GDH) based on immune-chromatographic mechanism. All participants in the study were examined using the RDT kit (Premier Medical Corporation Ltd., Mumbai, India).

Matching to the guidelines of the Malaria RDT kit provided by the company, five microliters of blood sample collected from participants' fingertip were added to a special well in RDTs kit. Afterwards three drops of lysing agent added to buffer well located on the on the RDT cassette to hemolysis the RBCs and release more parasite antigen. After 10–20 minutes, the results were evaluated according to the specific bands formation in the test and control windows (38).

Molecular Technique (Nested PCR)

The results of previous studies showed that routine diagnostic techniques (Microscopy and RDT) do not make enough sensitivity to diagnose asymptomatic malaria cases. Therefore, a robust and sensitive molecular method was used in this study beside the aforementioned methods. The molecular technique applied in this study was nested-PCR technique described by Snounou et al. with small sub-unit ribosomal ribonucleic acid (18 ssrRNA) of the parasite genome (39). Polymerase Chain Reaction (PCR) is a molecular laboratory technique that is implemented as an effective method to produce high amounts of specific DNA sequences using the some identified components in a buffer system (PCR Mix). The molecular detection was performed using nested-PCR to amplify the 18ssrRNA of the malaria parasite. In the beginning of method, the process of DNA extraction from 200 μL blood specimens was carried out by the Genomic DNA (Blood/ Culture Cell Mini Kit of "Yekta Tajhiz Azma" Iran Company. Briefly, 5μL extracted DNA in the first run was used, via primers targeting a specific region of *Plasmodium* genus that is common between species. In next step, second nest of experiment started by adding 2 μl of the first PCR product as a pattern for producing and multiplication of fragments of *P. vivax* (120 bp) and *P. falciparum* (205 bp) using internal primers. The Amplification was conducted in a final volume of 25μl including, 2–5 μl of templates, 250 nM of primers, 10mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 125 μM of each of the four deoxynucleotide triphosphates and 0.4 U of *Taq* polymerase (Invitrogen, Carlsbad, CA). First and second round repeated for 25 and 30 cycles, respectively while the annealing and extension temperature were 50 °C and 72 °C, respectively, for both reactions. (PCR machine, Apollo–ATC 401). Amplicon yields from the second round of PCR were analyzed in electrophoresis gel of 2% agarose. Results can be visualized under UV light. A sample would be considered as positive for *P. vivax* and *P. falciparum* if the 120 and 205 base-pair fragment appear, respectively. Positive and negative controls run in all series of PCR reactions.

For negative control, DNA samples have been taken from the blood of healthy individuals who did not previously experience malaria and had no previous travel history to endemic areas. Parasite DNA has been extracted from the blood of individuals who have had malaria confirmed by microscopic examination as part of a positive control preparation.

Results

The main characteristics of the participants were shown in Table 1. Of 246 studied cases, 56.5% were female and 43.5% were male. The age range was found to be 4 to 60 years old. Ninety-seven percent of the population was indigenous and the rest were immigrants.

Interestingly, despite the simultaneous use of Nested-PCR, microscopic and RDT meth-

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ods, malaria parasite was not recognized (Table 2). The robustness of microscopic experiments was assessed by reexamining the malaria blood smear in the reference laboratory of the School of Public Health. Positive and negative controls were used to guarantee the accuracy of the molecular results in each series of experiments. Altogether, these results indicate that there are no cases of asymptomatic malaria in the Anarestan region in 2018 (Table 2).

Table 1. Distribution of samples based on age groups, sex and Nationality in Kazerun County, Anarestan District, 2018

Variable	Category	No. (%)
Age Group	< 15 years	62 (25.2 %)
	15–30 years	65 (26.4 %)
	30–45 years	60 (24.4 %)
	> 45 years	59 (24 %)
Sex	Female	139 (56.5 %)
	Male	107 (43.5 %)
Nationality	Iranian	239 (97.2 %)
	Non-Iranian	7 (2.8 %)

Table 2. Comparison of methods evaluating the asymptotic malaria in Anarestan District, Kazerun County, 2018

Methods	Result	No. (%)
Microscopic	Positive	0 (0 %)
	Negative	246 (100 %)
RDT	Positive	0 (0 %)
	Negative	246 (100 %)
Molecular	Positive	0 (0 %)
	Negative	246 (100 %)

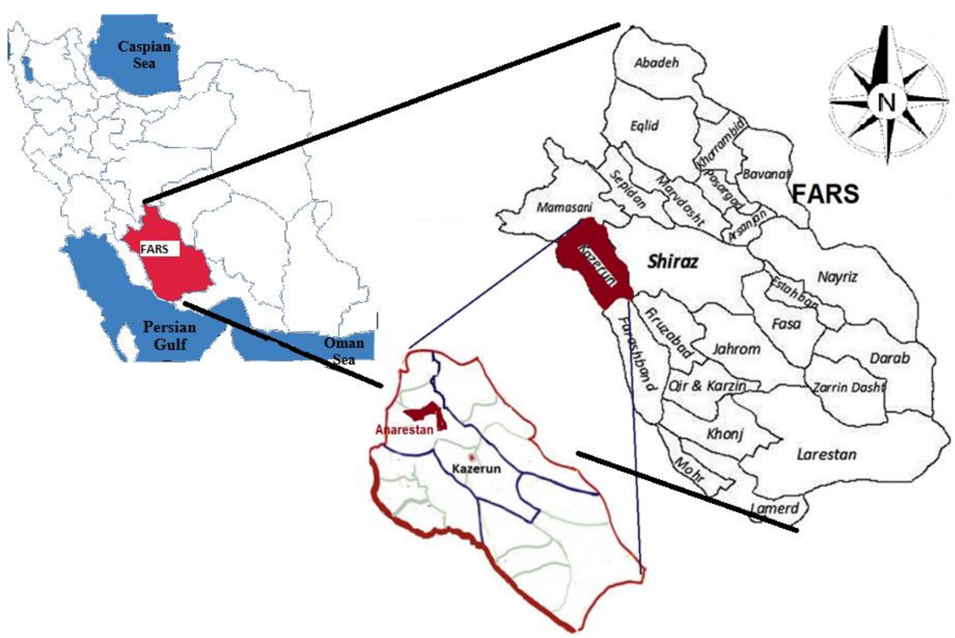

Fig. 1. Iran map indicating the study area; Fars Province, Kazerun County, Anarestan District (https://www.google.com/search =Kazerun+district)

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Discussion

The present research is the first study investigating malaria asymptomatic parasitic reservoirs in the Anarestan region with a previous history of local malaria transmission according to the report of Iranian Center for Disease Management and Control (CDMC). Although the robust and sensitive molecular techniques in addition to conventional malaria detection methods (Microscopic and RDT), asymptomatic cases have not been reported in Anarestan region. This result indicates the excellent performance of the Iranian Center for Disease Management and Control (CDMC) and Shiraz University of Medical Sciences in controlling and managing Anarestan local malaria transmission in 2016. Achieving an efficient MEP requires monitoring and detecting of asymptomatic cases of this disease. Asymptomatic malaria is considered as a hidden reservoir in the stable malaria transmission cycle and poses a significant challenge in implementing the MEP. The need to use sensitive molecular approaches to identify asymptomatic malaria infections and low- parasitemia has been emphasized by many researchers (40). The selection of practical and appropriate intervention strategies is essential for addressing MEPs in different regions. In addition to the determination of malaria transmission status and identification of parasitic reservoir in the studied area, prediction of the future situation of malaria in the region have been emphasized (41, 42).

This result match those observed in earlier studies conducted in Iran (8–13). Despite the simultaneous use of microscopic and sensitive molecular methods for diagnosis, no asymptomatic cases have been reported in these studies. The results of studies performed in Honduras, Sri Lanka and India are consistent with the results of the present study which no positive asymptomatic malaria cases have been reported (20–22).

However, the findings of the present study do not support a number of previous researches done in Iran (14–19) and some countries (23–32). The most important reason for the discrepancy between the results of this study and previous research may be the existence of a strong malaria monitoring system in the study area (Anarestan). This inconstancy in findings of the studies can be explained to some extent by differences in the ecological characteristics and epidemiological status of malaria in the study areas. Also, different political and economic situation of countries, the conditions of the health system and access to health facilities in the study areas, the genetic characteristics of the host, the displacement of human populations, the diversity of carriers, and their transmission capacity may justify the difference in the results (43).

The most important advantages of this study are considering both immigrants and natives for sampling, selecting the areas with a previous history of local malaria transmission, and using conventional and sensitive molecular techniques for diagnosis of malaria cases including microscopic, serological and Nested- PCR methods. The topic that was not considered in this study was the monitoring and follow-up of suspicious cases due to cross-sectional design of the study. To the best of our knowledge, this project was conducted comprehensively for the first time in Anarestan as an area with a history of local malaria transmission which could identify the challenges facing MEP in this area. The results of this study will facilitate the successful implementation of the MEP in Iran.

Conclusions

In the current study, asymptomatic parasitic reservoirs were investigated using sensitive molecular detection method along with routine malaria testing in the area with a previous history of transmission. In 2016, 12 cases of local transmission of malaria were reported in this area, mainly among foreigners. Despite the use of sensitive molecular method, the results of this study showed that there were no asymptomatic parasitic reservoirs in the study area. It can be

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concluded that the malaria elimination program is successfully implementing in Anarestan District, Kazerun County, without concerns about the asymptomatic parasitic cases. In addition, the findings indicated the existence of a strong malaria surveillance system in this area. Continuous studies in the high-risk area of malaria in Fars Province, especially in areas where foreign nationals are present, are recommended to monitor for malaria asymptomatic reservoirs.

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Ethical considerations

This research has been approved with ethics code of HUMS.REC.1396.155 in Hormozgan University of Medical Sciences.

Conflict of interest statement

The authors declare there is no conflict of interests.

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