Running title: Malaria detection in asymptomatic person

Identification and determination of malaria infection in asymptomatic person in endemic Area of southeast in Iran

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Abstract

Introduction: The persistence of asymptomatic malaria infections in endemic regions poses a major challenge for eradication efforts, as symptomless carriers can remain undetected, leading to continued transmission and misdiagnosis. This study addresses the prevalence of asymptomatic malaria carriers in Saravan and Suran districts, located in Sistan and Baluchistan Province, Iran.

Materials and Methods: A total of 985 samples were collected from asymptomatic individuals in Saravan and Suran districts between July 2019 and March 2024. Malaria infection was assessed using microscopic examination, ELISA, rapid diagnostic test (RDT), multiplex nested-PCR, and loop mediated isothermal amplification (LAMP) techniques. Participants were categorized by sex and age group to facilitate analysis.

Results: All samples tested negative for malaria infection using microscopy and RDT. ELISA identified the presence of antibodies against *Plasmodium falciparum* and *Plasmodium vivax* in 5 and 68 cases, respectively. Multiplex nested-PCR and LAMP techniques detected active *P. vivax* infection in 6 and 7 samples, respectively. The overall prevalence of malaria infection among sampled asymptomatic individuals was low.

Conclusion: Negative results from microscopy and RDT do not definitively exclude malaria infection due to limitations in sensitivity for low parasite levels. Although ELISA detected a greater number of antibody-positive cases, molecular diagnostic methods proved more effective in identifying current *P. vivax* infections, emphasizing their higher specificity for active parasitemia over historical exposure. Further molecular studies are recommended to accurately determine the prevalence and distribution of *P. falciparum* and *P. vivax* species within this population.

Keywords LAMP assay, RDT, *Plasmodium vivax, Plasmodium falciparum*

1. Introduction

Today, malaria is considered as a deadly infectious disease in developing countries with signs of chills, fever, and sometimes-fatal complications. In endemic areas of malaria, the clinical manifestations of *Plasmodium* infection vary from asymptomatic to severe and fatal. Malaria infection is categorized as one of the six significant diseases in WHO programs for tropical and subtropical area. In endemic areas of malaria, the high frequency exposure to malaria can lead to produce natural immunity against the clinical symptoms of the disease [1]. In addition, symptomless individuals provide the main reservoir of parasites and plays a role in sustaining malaria transmission [2]. To achieve a successful elimination program, all parasite carriers and active parasite cases must be identified and treated to stop malaria transmission in endemic areas. Symptomless malaria cases are described in areas with the high and moderate transmission, such as Ghana [3]. The asymptomatic malaria infection has reported from Asia, Africa, South America. It is commonly assumed that low-transmission settings have a smaller proportion of asymptomatic malaria cases compared with areas of higher transmission. However, community-based studies indicate that although higher transmission intensity increases the size of the parasite reservoir, asymptomatic infections still account for up to 60% of cases even in low-transmission regions. Consequently, malaria infections in low transmission settings are highly likely to be asymptomatic [4]. Some regions of Iran including, Sistan and Baluchestan, Kerman and Hormozgan provinces have the highest rate of malaria infection. The most important malaria control program in Iran is to eradicate of Malaria and the turn of Iran into a malaria-free zone in 1401 (2022) [5]. According to the latest WHO Global Malaria Report in 2020, there were approximately 241 million malaria cases with 627,000 deaths annually, which the number of deaths had increased by 69,000 more deaths compared to 2019. About 75% of these additional deaths were associated with impaired diagnosis, treatment and prevention strategies against malaria during the Corona pandemic [7] The primary purpose of this assay was to detection and prevalence of asymptomatic malaria infections in Saravan and Suran in Sistan and Baluchistan province using the microscopic, nested PCR, RDT, and Loop mediated isothermal amplification (LAMP). The results of this study could help the program to eliminate asymptomatic malaria in Sistan and Baluchestan province. The microscopic method as a standard gold method for the diagnosis of malaria is an easy, cheap and straightforward method. However, this test requires skilled operators,

especially when the parasitemia is low. Therefore, there is a need to find a new alternative diagnostic tool to malaria diagnosis [8]. Immunological methods such as rapid detection test (RDT) are time consuming, simple operation and storage compared to standard microscopy. The most important limitation of these methods is low sensitivity in cases where the number of parasites is less than 100-200 per microliter [9]. Conventional molecular tests, such as nested PCR, are susceptible and specific, even in detecting asymptomatic cases. The molecular tests can detect a variety of *Plasmodium* species. Most of the malaria-endemic countries have poor economic conditions and, cannot provide the equipment needed for molecular procedures [10]. LAMP is a molecular technique that is cheaper and simpler than conventional PCR [11]. One of the consequences of asymptomatic person is the persistence of reservoirs, which can interfere with, malaria eradication strategies. Therefore, the prevalence of symptomless malaria infection in different environments must be studied by appropriate identification methods. In order to facilitate and complete eradication efforts in Iran, this study was performed to evaluate the presence of asymptomatic parasitic carriers using microscopic methods, ELISA, RDT test, multiplex nested-PCR and LAMP in Saravan and Suran district in Sistan and Baluchistan province. The results of this study show the effectiveness and feasibility of interventions to eradicate malaria in endemic malaria areas in Iran.

2. Material and Methods

2.1. Area of study

The present study was performed in Saravan and Suran district in Sistan and Baluchistan province, during 2019-2024. Saravan city (27°22’15”N 62°20’3”E) with an elevation of 1195 m is located in the southeastern part of Zahedan. The population of Saravan is estimated at 60000. In general, Saravan city has a hot and dry climate with an average of 6.1mm annual rainfall. Malaria transmission is a year-round with two peaks during June to July and the September to October. The primary carrier of malaria is Anopheles stephensi[12]. The second study area was Suran city (27°17’8”N 61°59’47”E) in the southeast of Zahedan (Fig. 1) with a population of approximately 85095. In addition, its peaks are the same of Saravan city. Since 2009, malaria eradication programs have been launched in this area[13].

Figure. 1 The map of Iran showing the study area; Sistsn& Balouchestan province (A).. Saravan& Suran district.

2.2 Size calculation and sampling:

The openEpi was used for calculation of sample size with 95% confidence level. In previous study, the prevalence of symptomless malaria infection was reported 0% [14]. However, if assume that the minimum prevalence of symptomless malaria infection in any endemic area of malaria was 2% , the required sample size would be 45. In order to consider the missing data, the sample size increased to 985 samples. The participant signed the informed consent. The thick and thin blood smears were prepared from finger-prick for microscopy. However, for serological analysis and molecular tests blood samples were collected in tubes containing EDTA. Plasma was stored at -20 °C until use. All participants with history of malaria in the past ten years, history of anti malarial drug treatment in the last four weeks before and during follow-up, pregnancy, children below four years old and travel to endemic malaria areas in neighboring Afghanistan and Pakistan were excluded.

2.3Diagnostic tests:

2.3.1 Direct microscopic detection method

Blood samples were taken from the volunteers and smears were prepared from blood samples. Thin and thick slids were stained with Giemsa. All volunteers were tested for malaria.

2.3.2 ELISA method

The recombinant antigen of merozoite surface protein MSP-1-19KD were used for detection of malaria antibody with a final concentration of 2 μg/ml [15]. Then the plate was blocked with the blocking buffer (1% BSA in PBS 1X). The absorbance were measured using an ELISA plate reader (BioTek, USA) at 492 nm. The cut-off was calculated by mean of optical density of 30 samples from healthy individuals outside the endemic area of malaria plus three standard deviations.

2.3.3 Rapid Detection Test (RDT)

A commercially available RDT kit (Combo Rapid Diagnostic Test, INDIA) that used Plasmodium falciparum (pfHRP2) and lactate dehydrogenase (pLDH) as antigens was used to detect malaria parasites, according to the manufacturer's instructions. The strips of RDT kit were reported after 20 min, as described elsewhere [16]. The interpretation of the test results were made by two well-trained technicians.

2.4. DNA extraction

Genomic DNA extraction from whole blood was extracted by DNG-plus-kit method (Cinna Gene, Iran,) according to the instructions. This method is prepared in a tube containing EDTA (with final concentration of 1 mg/ml) to prevent DNA damage.

2.5. Multiplex/nested PCR

Three pairs of primers (Table 1) were used for Nested PCR and determination of *Plasmodium* species.

Table1. Primers for NESTED-PCR to determine Plasmodium species

Reference name	Product length	Species	Sequence	Primer
22	12000bp		CTTGTTGTTGCCTTAAACTTC	Rplu5
			TTAAAATTGTTGCAGTTAAAACG	rplu6
22	205bp		TTAAACTGGTTTGGGAAAACCAAATATATT	rFAL.1
			ACACAATGAACTCAATCATGACTACCCGTC	
			rFAL.2	P. falciparum
22	120bp		CGCTTCTAGCTTAATCCACATAACTGATAC	rVIV.1
			ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	
			rVIV.2	P.vivax

Three pairs of primers were used for Multiplex / nested PCR assay to determine the Plasmodium species. A genus of *Plasmodium* were identified by specific primers (rPLU6 and rPLU5) at first round of PCR. In the first round of amplification, the PCR program was performed as fallow: 5 min at 95°C followed by 35 cycles of 45 s at 95°C, 45 s at 58°C, and 60 s at 72°C, with a final extension of 5 min at 72. In PCR-2, the species were determined by two pair primers (rVIV.1, rVIV.2 for *P. vivax* and rFAL.1, rFAL.2 for *P. falciparum*). Thermal parameters in this steps was performed as follow; 5 min at 95°C followed by 30 cycles of 45 s at 95°C, 25 s at 56°C, and 45 s at 72°C, with a final extension of 5 min at 72°C Amplified sequence were account as a positive for *P. falciparum* and *P. vivax* if standard band markers a 205 and 120 base-pair product was visualized, respectively [5-17].

2.6. LAMP technique

The LAMP primer sets, as previously described, were used to amplify the gene coding for the 18S rRNA of Plasmodium genus. 3 Each LAMP reaction mixture contained 2 mL of both FIP and BIP primers, 0.25 mL F3 and B3 primers, 1 mL LPF and LPB, 1 mL of Bacillus stearothermophilus DNA polymerase, 2 mL MgSO4, 4 mL betaine, 3.5 mL Dntp mix and 1 mL of DNA sample in a total volume of 25 mL. In order to set up the LAMP technique and determine the best temperature and time conditions for it, the reaction in the gradient thermocycler was in the range of different temperatures from 60 to 67 ºC and 30, 45, 60, and 75 minutes. A LAMP reaction was considered positive for *Plasmodium* spp. If an apparent increase in the turbidity was observed by the naked eye compared with the negative control. The results were evaluated blindly by two researchers [17-18].

3. Results

To assess the presence of asymptomatic malaria infection in Saravan and Suran district, 985 volunteers with no symptoms of malaria were selected in this cross-sectional survey. All study samples were categorized by sex and age group (Table 1). Sampling was performed during July 2019 to March 2024. Most samples were aged 41-20 years (38%). All 985 samples of thick and thin Giemsa-stained blood smears were examined for detecting *Plasmodium* spp. and no *Plasmodium* parasites were found in the samples. In ELISA method, 68 out of 985 samples (6.9%) had specific IgG antibodies against rPvMSP 1_{42} of *P. vivax* antigen. The positive cases from Saravan and Suran districts were 41 and 27, respectively. In serological results using rPfMSP-1_{19} as anti-*P. falciparum* antigen, 5 out of 985 samples (0.5%) had IgG-specific anti-rPfMSP-1_{19}KD antibodies, among which 2 person was related to Suran city and others from Saravan district. The cut-off value for ELISA tests were calculated as 0.388 and 0.53, for *P.vivax* and *P. falciparum*, respectively. The RDT was performed by PLDH / HRP2 Combo Rapid Diagnostic Test (India) for all 985 samples. Reading and interpretation of test results was performed by two expert technicians and coordinated in two tests of 15-30 minutes. No *Plasmodium* species were found using RDT assay. The identification of 18ssrRNA of *P. vivax* and *P. falciparum* were performed by multiplex/nested PCR, in which, only 6 samples of *P. vivax* related to Saravan district were positive and no positive cases were recorded for *P. falciparum* (Figure. 2).

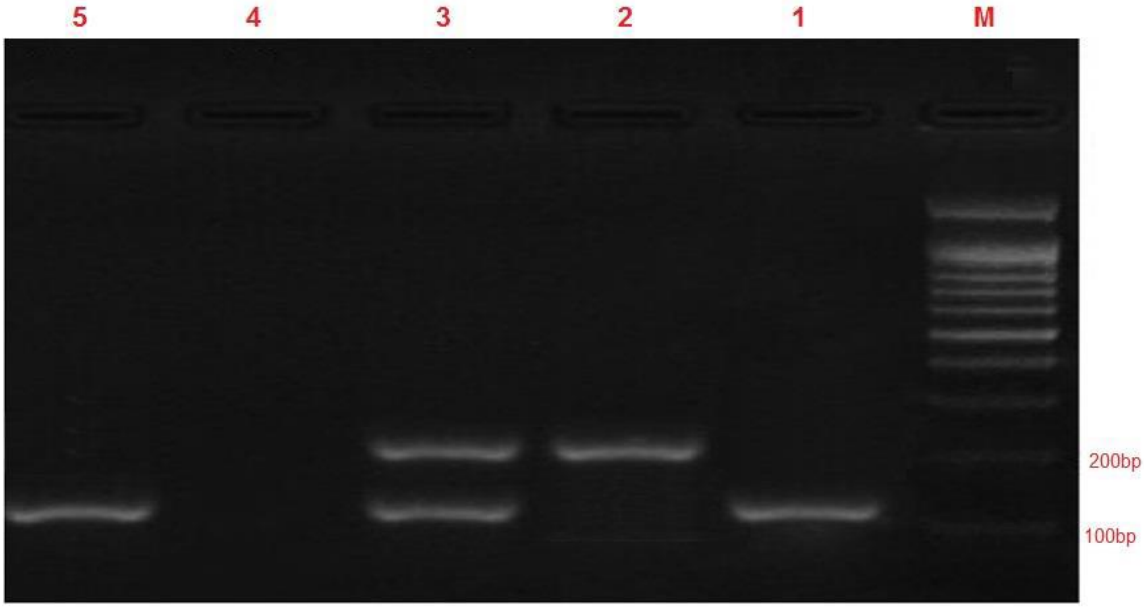

Figure. 2 Electrophoresis of the ssrRNA gene multiplex/nested PCR products on a 2% agarose gel, M: DNA marker (ladder) used as a size reference to estimate the approximate length of PCR products in base pairs (bp). Lane1: Positive control of *Plasmodium vivax* (120 bp), Lane2: Positive control *of Plasmodium falciparum* (205 bp), Lane3: Positive control of mixed infections, Lane4: Negative sample, Lanes 5: Positive sample of *P. vivax*. M: Ladder 100 bp

The LAMP method was performed to identify the 18SSU rRNA genomic target of *P. vivax* and *P. falciparum* at 60 ° C for 90 minutes. In this test, seven samples of *P. vivax* were detected, in which, six of them was related to Saravan and another was from Suran. six of them were positive in multiplex/nested PCR. No positive case was found for *P. falciparum* (Figure. 3).

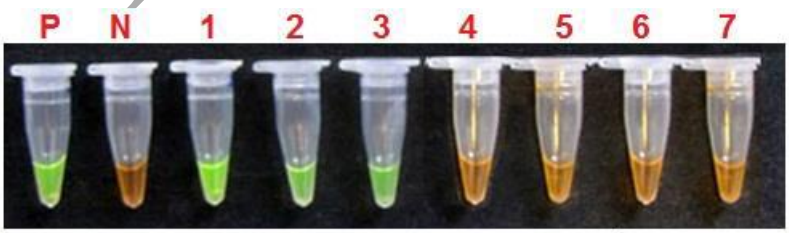

Figure. 3 LAMP technique; The LAMP products was visualized with SYBR Green. P: 188.positive control, N: negative control

4. Discussion

The identification of active and asymptomatic reservoirs of malaria cases is one of the most essential strategies in malaria eradication program (15). Asymptomatic malaria occurs in people with low levels of parasitemia who have no clinically significant symptoms. So, these people are not treated and may act as a reservoir of disease in the area [19]. Considering the role of asymptomatic malaria in malaria eradication programs, the primary goal of the current study was to monitor the prevalence of symptomless cases of malaria infection in Saravan and Suran in Sistan and Baluchestan province. Based on multiplex/nested PCR test, six people who had high antibody titers were positive for *P. vivax*. In LAMP test, seven people were positive for *P. vivax* with high antibody titers. According to the report (CDMC 2022), the number of positive cases in Saravan city decreased from 456 people in 2008 to 156 people in 2020 and Suran city from 69 people in 2008 to 15 people in 2020, which shows a significant reduction in malaria in these areas. However, in Saravan and Suran, with low prevalence of *Plasmodium* infections, they still need to strengthen and maintain optimal monitoring. These cities have a favorable climate for the life of malaria-carrying mosquitoes, Neighborhood with Pakistan and Afghanistan and malaria contamination in these countries, frequent border traffic on both sides of the border with Pakistan, fuel smuggling and the entry of disease parasite reservoirs, recent rainfall and floods in Pakistan and the border cities of Sistan and Baluchestan provinceand high population density of malaria-carrying mosquitoes. In this study, multiplex/nested PCR and LAMP methods were used due to their high sensitivity in addition to the microscopic and RDT tests. The results of current study is consistent with previous studies that performed in Iran, including the cities of Minab [20] and Bashagard [21]. An important reason for the discrepancy between this study and previous studies may be using highly susceptible diagnostic methods for malaria. In addition, there are similarities between this study and similar studies conducted in other countries by researchers in Yemen [22], Solomon Islands [23] and Paraguay [24]. However, there are findings contrary to studies in India [25]. Where asymptomatic malaria has not been reported. The main strength of this study is the use of various technique including microscopy, serological and molecular techniques for malaria

diagnosis. This study showed that a significant challenge to eliminate of malaria in Saravan and Suran districts is the presence of asymptomatic individuals who carry the parasite gametocyte in their bodies.

Conclusion

The current study showed that sensitive and accurate diagnostic methods of malaria along with the usual diagnostic methods of microscopy and RDT are necessary to stop the transmission of malaria in Iran. To improve the sensitivity, specificity and high reliability of malaria removal in the country, especially in endemic areas, all sensitive tools such as microscopy RDT, LAMP and nested PCR should be used.

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Ethical approval

All individuals were interviewed for sampling and after their clinical examinations, consents were reviewed. This study has been approved by the Research Ethics Committee at Zahedan University of Medical Sciences IR.ZAUMS.REC.1397.027. All authors read and approved the final manuscript.

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Study concept and design: M.H, R.S, H.Y

Acquisition of data:,M.H, H.Y

Analysis and interpretation of data: R.S &M.H

Drafting of the manuscript: H.Y

Critical revision of the manuscript for important intellectual content: R.S

Statistical analysis: M.H

Administrative, technical, and material support:M.H

Data Availability Statement:

The data that support the findings of this study are available upon request from the corresponding author

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