Monitoring of *Plasmodium* infection in humans and potential vectors of malaria in a newly emerged focus in southern Iran

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ABSTRACT

Despite control programs, which aim to eliminate malaria from Iran by 2025, transmission of malaria has not been removed from the country. This study aimed to monitor malaria from asymptomatic parasitaemia and clinical cases from about one year of active case surveillance and potential vectors of malaria in the newly emerged focus of Mamasani and Rostam, southern Iran during 2014–2015. Samples were collected and their DNAs were extracted for Polymerase Chain Reaction (PCR) assay using specific primers for detection of *Plasmodium* species. The Annual Parasite Incidence rate (API) was three cases per 1,000 population from 2,000 individuals in three villages. Parasites species were detected in 9 out of the 4,000 blood smear samples among which, 6 cases were indigenous and had no history of travels to endemic areas of malaria. Also, the prevalence rate of asymptomatic parasites was about 0.3%. Overall, 1073 *Anopheles* spp. were caught from 9 villages. Totally, 512 female samples were checked by PCR, which indicated that none of them was infected with *Plasmodium*. Despite new malaria local transmission in humans in Mamasani and Rostam districts, no infection with *Plasmodium* was observed in *Anopheles* species. Because of neighboring of the studied area to the re-emerged focus in Fars province (Kazerun) and important endemic foci of malaria in other southern provinces, such as Hormozgan and Kerman, monitoring of the vectors and reservoir hosts of *Plasmodium* species would be unavoidable. Application of molecular methods, such as PCR, can simplify access to the highest level of accuracy in malaria researches.

KEYWORDS

Plasmodium; Human; *Anopheles*; PCR; Malaria; Iran

Introduction

Malaria, as one of the most important vector-borne diseases, is one of the major health problems and factors of morbidity in the world [1]. Based on the annual reports of malaria by the World Health Organization (WHO), millions of cases of malaria have occurred in 106 countries around the world, with more than 80% of the victims being below-5-year-old children [1,2]. Thus, it has been considered to be a major barrier to development and economic improvement of human communities [2]. On the other hand, malaria affects individuals' lives by increasing poverty, limiting education opportunities, and causing absenteeism from school or work [2]. Nowadays, malaria is the most important parasitic disease in tropical and subtropical areas as well as in Iran and mainly involves the southern and south-eastern regions of the country [3]. Recently, because the annual cases of malaria have decreased from more than 5,594 to 366 during 2008–2014 [4], the national malaria strategic plan has aimed to manage malaria by distribution of Long-Lasting

Insecticidal Nets (LLINs), application of larvicides, and/or Indoor Residual Spraying (IRS) [5]. Although Iran has reached the elimination stage, malaria is still present in some provinces [6]. However, Iran is targeting to become a malaria-free area by 2025 [7].

Mosquitoes control is an important method in the global malaria control program and has been considered to be the most effective measure for eradication and reduction of malaria transmission at the public level in different parts of the world [8]. World climatic changes associated with human activity, which affect development and survival rate of the malaria vectors may change anopheline species composition [9]. Thus, a full understanding of environmental aspects can be a main factor in malaria control strategies. Therefore, it is vital to understand the distribution and infection of anopheline mosquitoes in different areas before management of any vector control programs [10].

In Iran, studies on anopheline mosquitoes have reported 34 *Anopheles* species in two subgenera of *Anopheles* and *Cellia* (including genotypes and sibling

species), eight of which being involved in malaria transmission [11]. Also, 22 out of these 34 species are in complexes or sibling species, some of which are known as malaria vectors [11]. The sibling species are as follows: 1. *An. plumbeus* (in *An. plumbeus* complex), 2. *An. sacharovi*, 3. *An. maculipennis*, 4. *An. messeae*, 5. *An. martinus*, 6. *An. labranchiae*, 7. *An. melanoon*, 8. *An. persiensis*, 9. *An. atroparvus*, and 10. *An. marteri* (in *An. maculipennis* complex), 11. *An. pseudipictus*, 12. *An. hyrcanus*, 13. *An. peditaeniatus*, and 14. *An. nigerrimus* (in *An. Hyrcanus* group), 15. *An. culicifacies* A (in *An. culicifacies* complex), 16. *An. fluviatilis* T, 17. *An. fluviatilis* U, and 18. *An. fluviatilis* V (in *An. fluviatilis* complex), 19. *An. superpictus* X, 20. *An. superpictus* Y, and 21. *An. superpictus* Z (in *An. superpictus* complex), and 22. *An. subpictus* (in *An. subpictus* complex:) [11]. Species, such as *Anopheles dthali* Patton, *An. fluviatilis* James s.l., *An. superpictus* Grassi s.l., *An. culicifacies* Giles s.l., and *An. stephensi* Liston, were reported as the proven vectors of malaria in southern areas [12,13]. Moreover, *An. pulcherrimus* Theobald sporozoite infection was reported from south-eastern areas [14]. In western, north-western, and central plateau areas also, *An. maculipennis* s.l., *An. sacharovi* Favre, and *An. superpictus* were introduced as the vectors of malaria [15]. In addition, molecular infection of *An. hyrcanus* Pallas and *An. subpictus* Grassi with *Plasmodium* was reported from northern and south-eastern corners of Iran, respectively [16,17]. Generally, information about fauna, distribution, and infected species of anopheline mosquitoes in different areas plays a specific role in monitoring and planning effective control measures for malaria elimination.

Up to now, different biochemical, molecular, and immunological methods, such as iso-enzyme electrophoresis, Polymerase Chain Reaction (PCR), and monoclonal antibodies, have been used to characterize the causative agents of malaria [18,19]. PCR has been shown to be more sensitive and specific compared to microscopic assay, which can help detect infection in cases of low parasitemia and mixed infections [18]. Yet, using a specific and sensitive PCR on human and the anopheline mosquitoes would be beneficial to detect the *Plasmodium* species accurately [18,19].

Sistan and Baluchistan, Hormozgan, and southern part of Kerman provinces are the most important endemic areas of malaria transmission in Iran [3]. These provinces have been limited by Persian Gulf and Oman Sea and are located in neighborhood of Afghanistan and Pakistan [13]. Besides, they comprise 12% of Iran's population and account for 62% of the country's malaria cases [4]. Fars is Hormozgan and Kerman's neighboring province and communication and inter-provincial travels in these regions, especially migration of foreign workers, could increase the probability of malaria transmission in this province [20]. Fars province is a malaria endemic focus in south of Iran and local transmission occurs in southern regions of this province [20,21]. Thus, mosquito

control strategies, including larviciding, IRS, and LLINs, have been applied as a part of an integrated approach to malaria elimination in southern areas of the province [3]. Despite these human interventions, malaria is present in some parts of Fars province, such as Mamasani and Rostam located near the re-emerged focus of Kazerun (Figure 1).²⁰ Because of the suitable environmental conditions for malaria, such as existence of anopheline mosquitoes, and imported cases of malaria in Mamasani and Rostam, it is essential to monitor and check human and anopheline mosquitoes' infection with *Plasmodium* spp. in specified periods annually. Therefore, this study aimed to provide good information on human infection cases and distribution and infection of anopheline species with *Plasmodium* spp. in different parts of Mamasani and Rostam near the re-emerged focus of malaria in Kazerun, south of Fars province. The results of this study will offer information that would help plan and implement effective strategies for vectors control during the elimination phase by the National Malaria Control Program.

Materials and methods

Study area

Mamasani district is located in the highlands of Fars province, situated 900 meters above the sea-level, and distanced 158 km from Shiraz (31°13' N, 51°32' E). It consists of two districts, namely Mamasani and Rostam with capitals of Nourabad and Masiri, respectively. Besides, it is bound by Sepidan in the north, Bushehr and Kohkiluyeh-Boyerahmad provinces in the west, Kazerun and Bushehr province in the south, and Shiraz in the east (Figure 1) [22].

Human active case surveillance

Asymptomatic Parasite Screening (APS) was conducted from March to April and from June to July in 2014. The participants were selected randomly among the 2907 individuals in Moraskhun, Kopen-Olia, and Kopen-Sofla villages in Rostam district. Smear blood samples were obtained in two stages. Overall, 4,000 thin and thick blood smears were prepared for laboratory examination by the standard finger-prick method [23]. All the slides were examined by two experienced laboratory technicians at Centre for Disease Control (CDC) of Shiraz University of Medical Sciences to identify the parasite species. For quality control, a third technician was selected to re-examine 5% of the slides.

Clinical malaria cases were defined as individuals with malaria-related symptoms, such as fever, body temperature more than 37.5 °C, chills, severe malaise, headache, and vomiting, at the time of examination or one to two days prior to examination, with a *Plasmodium*-positive blood smear. For the participants with signs of malaria, blood samples were taken to prepare a thin and a thick

Figure 1. The geographical coordinates of Iran, including the studied villages of Mamasani and Rostam districts; Pereshkaft (No. 1), Moraskhun (No. 2), Kopen-Olia (No. 3), Jowzar-Javid (No. 4), Pirsirkeh (No. 5), Cham-Gaz (No. 6), Bidkarz (No. 7), Milatun (No. 8), and Razak (No. 9).

smear for parasite species identification [22]. Blood smear tests were conducted in the hospital and were confirmed in the CDC laboratory in Shiraz University of Medical Sciences. The clinical cases were referred to the hospital for free treatment. The active case surveillance was followed from March 2014 to January 2015. Moreover, the positive cases were selected for PCR assays.

Mosquito samples collection

During April 2014 to January 2015, a total of 1073 adult anopheline samples were collected from different regions of Mamasani and Rostam districts. Several regions from the mountainous and plateau villages of Milatun, Bidkarz, Pirsorkh, Pereshkaft, Jowza-Javid, Razak, Moraskhun, Kopen-Olia, and Cham-Gaz were selected for the survey. Rural districts of the studied area were also checked monthly for their anopheline fauna during their activity season. This was continued for over 9 months. Pyrethrum space spray total collection was implemented in four human and four animal indoors in different localities of each village [24]. In doing so, windows and doors were closed and floors were shielded with a white sheet. Then, the killed mosquitoes by aerosol pyrethrum of 0.2% were collected during 6 to 10 AM [25]. Outdoor studies were also performed to catch anophelines using night catch, CDC light trap, and suction tube [23]. Afterwards, anophelines mosquitoes were transferred to the laboratory, stuck, and identified by systematic keys [26,27]. Female samples of *Anopheles* spp. were also detected by the characteristic key selected for PCR diagnostic assay.

PCR assay

PCR was performed on all the collected female samples. In doing so, DNAs of the anopheline tissues were extracted by digestion in 1.5 mL micro-centrifuge tubes with 200 μ L lysis buffer (1 mM ethylene diamine tetra acetic acid, 50 mM Tris-HCl (pH 7.6), 1% Tween 20 (v/v)) containing 5 μ L of proteinase K solution. The tubes were incubated at 56 $^{\circ}$ C for 2 h before addition of 200 μ L phenol/chloroform/isoamyl alcohol (25:24:1, by vol). The tubes were then shaken vigorously and centrifuged at 12000 rpm for 10 min. The resultant supernatant solutions were mixed with 300 μ L cold absolute ethanol. The precipitated DNAs were centrifuged down (at 12000 rpm for 10 min), dried, dissolved in 100 μ L deionized distilled water, and stored at -20° C until analysis [20,28]. In the initial amplification reaction, the primer pair rPLU5 (5-CCT GTT GTT GCC TTA AAC TTC-3) and rPLU6 (5-TTA AAA TTG TTG CAG TTA AAACG-3) was used [29]. The size of the DNA target amplified by these outer primers was about 1200 bp. These primers were genus specific and could, therefore, amplify the target sequences from all the four species of *Plasmodium* spp. (*P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*) [30]. The second reaction was performed for specific detection of *Plasmodium* spp. using a set of two primer pairs; i.e. rFAL1 (5-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3) and rFAL2 (5-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3) for *P. falciparum* and rVIV1 (5-CGCTTCTAGCTTAATCCACATAACTGATAC-3) and rVIV2 (5-AAG GAA AGA AAG TCC TTA-3) for *P. vivax* [20]. The size of the DNA target was about 120 and 205 bp for *P. vivax* and *P. falciparum*, respectively. For

each series of reactions, the researchers made use of 20 µL of the reaction mixture for the first amplifications containing 5 µL of template DNA, 2 µL of 10x PCR buffer (50 mM KCl, 10 mM Tris-HCl), 4 mM MgCl₂, 200 µM of each dNTPs, 0.4 units of Taq DNA Polymerase, and 250 nM of each primer (rPLU5 and rPLU6). The PCR settings were as follows: 95 °C for 5 min, 25 cycles at 94 °C for one min, 58 °C for two min, extension at 72 °C for two min, and final extension at 72 °C for 7 min. About 2 µL of the first amplification products served as the DNA template for each 20 µL of the second PCR amplification. The concentrations of the second primers and other constituents were identical to the first amplification, except for using 0.3 unit of Taq DNA polymerase [31]. After all, the PCR products were subjected to gel electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and visualized under Ultra Violet (UV) light [18,32].

Results

Totally, nine malaria cases were detected in the human APS and clinical studies. In the APS study, 4,000 blood samples were obtained and parasites were detected in 6 cases (0.3%); one in Moraskhun (per 657 individuals), two in Kopen-Olia (per 2000 individuals), and three in Kopen-Sofla (per 250 individuals). These cases included a 45-year-old woman and 5 men aging 25, 28, 32, 34, and 43 years. They were referred to therapeutic centres to follow up their infection and were discharged after receiving Chloroquine and Primaquine chemotherapy treatments. Moreover, three cases were identified in the clinical study, but they had a history of traveling to other malaria endemic areas. All these nine cases were assessed using PCR assay and the results revealed all to be *P. vivax* (Figure 2).

In vector studies, out of the 1073 samples, 766 *Anopheles dthali*, 153 *An. superpictus*, 153 *An. sacharovi*, and one *An. stephensi* were collected from 9 villages in Mamasani and Rostam districts (Table 1).

PCR results revealed no infections of female *Anopheles* spp. with *Plasmodium* spp. (Table 2).

Figure 2. The results of PCR assay on human samples stained with ethidium bromide on a 1.2% agarose gel; *P. vivax* reference control (Lane 1), negative control (Lane 2), positive human samples (Lanes 3–6), and molecular marker (100 bp Cinnagen, Iran) (Lane 7).

Table 2. *Anopheles* species collected and evaluated for PCR assay in Mamasani and Rostam districts.

Genus and Species	No. of			PCR results	
	Male	Female	Total	Positive	Negative
<i>Anopheles dthali</i>	423	343	766	0	343
<i>Anopheles superpictus</i>	27	126	153	0	126
<i>Anopheles sacharovi</i>	111	42	153	0	42
<i>Anopheles stephensi</i>	0	1	1	0	1
Total	561	512	1073	0	512

Table 1. Geographical distribution of *Anopheles* caught in Mamasani and Rostam districts during April 2014 to January 2015.

Villages	Geographical status	No. of				
		<i>Anopheles dthali</i>	<i>An. superpictus</i>	<i>An. sacharovi</i>	<i>An. stephensi</i>	Total
		Outdoor/Indoor	Out./In.	Out./In.	Out./In.	Out./In.
Milatun	Plateau	0/0	10/2	0/0	0/1	10/3
Bidkarz	Plateau	20/4	2/0	6/0	0/0	28/4
Razak	Plateau	0/0	15/0	5/0	0/0	20/0
Moraskhun	Plateau	211/38	30/3	38/9	0/0	279/50
Kopen-Olia	Plateau	194/53	25/9	49/16	0/0	268/78
Cham-Gaz	Mountainous	104/45	4/0	22/8	0/0	130/53
Pirsorkh	Mountainous	7/2	0/0	0/0	0/0	7/2
Pereshkaft	Mountainous	14/5	7/5	0/0	0/0	21/10
Jowzar-Javid	Mountainous	58/11	35/6	0/0	0/0	93/17
Total (%)		608/158	128/25	120/33	0/1	856/217
		766 (71.39)	153 (14.26)	153 (14.26)	1 (0.09)	1073 (100)

Discussion

Totally, nine malaria cases were detected by microscopic and PCR assays in the human APS and clinical studies in Mamasani and Rostam districts. In other studies, different methods were used to detect malaria cases. For example, 11 *P. vivax* mono-infection cases, which were indigenous, were detected by microscopic examination in Bashagard district in Hormozgan province in 2010 [33]. Moreover, 13 malaria cases were reported from Ghale-Ganj district in Kerman province, nine of which were indigenous [2,33]. Similarly, Zakeri et al. (2002) carried out a research on blood samples of 120 volunteers seeking for care at the Malaria Health Centre in Chahbahar district [34]. The results indicated that 107 out of 120 Giemsa-stained slides were positive for malaria parasites by microscopy. Besides, 84 and 20 samples were identified as having only *Plasmodium vivax* and *Plasmodium falciparum*, respectively [34]. On the other hand, only three samples were shown to have mixed *P. vivax* and *P. falciparum*. Furthermore, nested PCR assay detected 31 more mixed infections compared to microscopy. Also, the results confirmed the presence of *P. vivax* and *P. falciparum* in the blood samples [34].

According to the Iranian Centre for Diseases Management and Control (CDMC), because of applying massive malaria control tools, the prevalence and incidence of malaria cases have recently reduced in endemic regions in southern Iran. This has also been confirmed in the recent studies conducted on the issue [33]. Moreover, APS emphasized asymptomatic infections, which are usually hidden parasite reservoirs [35]. As the result of passive and active case detection and evaluation of malaria surveillance and control interventions in Iran, the prevalence and incidence of malaria cases have decreased since 2008 [33]. In endemic areas, continuous exposure to parasites leads to asymptomatic carriers that provide an essential reservoir of parasites, contributing to persistence of malaria transmission [33]. Moreover, low asymptomatic carriers with low PCR-positive cases to *P. vivax* and non-positive cases to *P. falciparum* can explain the limited recent transmission in the studied areas. Yet, continued follow-up and action are still needed in these area [33]. In the present survey, the presence of malaria asymptomatic cases was determined to evaluate the reservoirs of infection in two newly emerged foci (Mamasani and Rostam districts) with a previous history of malaria transmission in south of Iran. The study results indicated that surveillance devices revealed different epidemiological profiles. Indeed, a recent study confirmed that clinical reports severely underestimated the morbidity and mortality of malaria [35].

In the vector survey in the current study, four anopheline species were identified in Mamasani and Rostam districts, namely *An. dthali*, *An. superpictus*, *An. stephensi*, and *An. sacharovi*, which are known as malaria vectors in Iran. *Anopheles stephensi* is considered to be the main

vector and *An. dthali* and *An. superpictus* play the key role as the secondary vectors in south and southeast of Iran [36,37]. *Anopheles dthali* was collected in Mamasani and Rostam districts plentifully. This is consistent with the results of former studies, indicating that *An. dthali* was well distributed in southern areas of Zagros mountain chains up to about 1,400 meters above the sea level [36]. This anopheline species has been known as a minor vector in southern areas of Iran as well as in northern regions of Saudi Arabia in south-east of Asia and Somalia [37]. Despite its probable contribution to malaria transmission with sporozoite rate of about 0.5–2.5 [37], it is unlikely that this species could play an important role in malaria transmission in the absence of the major vectors in the areas under the present investigation.

Anopheles superpictus was also collected from all regions of the studied area. Variant sibling species of *An. superpictus* X, Y, and Z have been introduced as vectors in central and southern areas of Iran [36,38,39]. It is in fact the most prevalent anopheline in Iran and survives in all parts of the country up to 2,000 meters in any circumstances, including arid to wet climates [39]. On the other hand, it has an extensive geographical distribution in Asia, Europe, and northern Africa and has been introduced as a malaria vector in these regions [36].

Anopheles sacharovi Favre was introduced as the vector of malaria in the central plateau, western, and north-western areas [15]. Moreover, Yaghoobi-Ershadi et al. (2001) studied the bionomics of this species in Ardabil province and introduced it as the new epidemic focus of malaria in northeast of the country [40]. Because the high dominance of *An. sacharovi* was revealed in Azerbaijan, Oshaghi et al. (2011) also suggested the possible danger of malaria epidemic in the region, which needed for a continuous epidemiological care [41]. Due to immigration of infected individuals between Azerbaijan republic and these areas of Iran, quarantine procedures were effective in malaria control program [12]. Phylogenetic tree-based study of *An. maculipennis* complex revealed that the six Iranian members of the maculipennis complex could be easily clustered into three groups: *An. atroparvus*, *Anopheles labranchiae* (*An. maculipennis*, *An. messeae*, and *An. persiensis*), and *An. sacharovi* groups [42,43]. Detection of three species of *An. atroparvus*, *An. messeae*, and *An. labranchiae* in the *An. maculipennis* complex, as new records, is alarming in epidemiology of malaria on the sides of the Caspian Sea in northern Iran [44].

Anopheles stephensi Liston is the most prevalent anopheline species and plays an important role in transmission of malaria in Indian subcontinent and Middle East including southern parts of Iran [45]. It is the main vector in seaside areas of south of the country and its infection with *P. vivax* and mixed infection with *P. vivax* and *P. falciparum* have been reported in these areas [46]. It is also an important vector in Hormozgan

province where the most prevalent anopheline mosquito exists [46]. In terms of ecology, it has two annual activity peaks in spring and autumn. In mountainous regions, the autumn peak begins sooner than in coastal regions [47]. Adults of *An. stephensi* tend to be endophilic and endophagic, but they often bite outdoors in hot months of the year when people sleep out [47]. Furthermore, larval habitats are wide-ranged in both nature and man-made furniture. Different biological forms of the species (*mysorensis*, type, and intermediate) occur in Hormozgan province [45]. Existence of the type form in urban and *mysorensis* form in rural regions confirms the epidemiological data regarding malaria in the region. It is also consistent with the results obtained in India indicating that the type form was an efficient malaria vector inhabiting in urban regions, whereas *mysorensis* was rural and had a lower vectorial capacity [45,47].

In conclusion, although new malaria local transmission has occurred in Mamasani and Rostam districts, no infection with *Plasmodium* was detected in *Anopheles* species. Due to neighboring of this area to the re-emerged focus in Fars (Kazerun) and important endemic foci of malaria, such as Hormozgan and Kerman, monitoring of the vectors and reservoir hosts of *Plasmodium* species would be inevitable. Furthermore, application of PCR can facilitate quicker access to the highest level of accuracy in malaria researches.

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