Original Article

Application of High-Resolution Melting (HRM) Technique towards the Detection of Asymptomatic Malaria in a Malaria Endemic Area of Southeastern Iran under Elimination Program

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Abstract

Background: Asymptomatic malaria, which usually exists in low parasitemia, acts as the Plasmodium species reservoirs contributing towards malaria transmission. This situation hinders malaria elimination programs in endemic areas, thus necessitating an active case detection with a high sensitive method and treatment of cases. This is why we used a High Resolution Melting (HRM) assay to monitor the trend of asymptomatic malaria in a malaria endemic area of Iran which is under elimination program.

Methods: The peripheral blood was sampled from 271 clinically approved non-febrile individuals from a malaria endemic zone of southeastern Iran for asymptomatic malaria prevalence detection by microscopy, Rapid Diagnostic Tests (RDTs) and HRM methods. The HRM assay was done based on the amplification of 18S SSU rRNA gene.

Results: The HRM assay revealed infections from three individuals out of 271 (1.1% asymptomatic malaria prevalence) from the participants, two Iranian natives with *Plasmodium vivax* infection and one Pakistani immigrant with *P. falciparum* infection. Neither microscopy nor RDTs detected *Plasmodium* spp infections from the 271 non-febrile individuals. The nucleotide sequencing analysis of the positive controls used in this study showed a close homology with the reference gene bank sequences of *P. falciparum* 3D7 (CPO16995.1) and *P. vivax* Sal-1(UO3079.1).

Conclusion: This study revealed a low frequency of asymptomatic malaria trend within malaria endemic areas of southeastern Iran which are under intense elimination program and also the ability of HRM assay in detecting low *Plasmodium* spp parasitemia beyond the limits of microscopy and RDTs.

Keywords: Asymptomatic malaria; High resolution melting (HRM); Microscopy; Rapid diagnostic test (RDTs); Iran

Introduction

Malaria remains an infection of global concern with almost half of the world's population being at risk (1, 2). The disease burden remains high, in spite of 21% and 29% reported reduction in prevalence and deaths respectively between 2010 and 2015 (3, 4). The estimated global malaria cases and deaths in 2018 stood at 228 million people and 405,000 people respectively. Malaria annual global funding estimate stands at US Dollars 2.7 Billion (4).

Iran, which is in malaria elimination phase, reports a low malaria prevalence (of 0.87%) with the most endemic areas (including Sistan and Baluchestan, Kerman and Hormozgan Provinces) being in the south and southeastern part of the country where most cases are imported from malaria endemic neighboring countries of Afghanistan and Pakistan (5).

The continuous exposure of human to malaria parasites in malaria endemic areas may create asymptomatic carriers which act as parasite reservoir harboring gametocyte and contribute towards malaria transmission (6). A severe malaria disease in these areas occurs most-

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ly in non-immune individuals such as children or visitors. Asymptomatic malaria is the malaria parasitemia of any density without any ordinary symptom in an individual who has not received any recent antimalarial treatment. Asymptomatic malaria results from a partial immunity which only arrests but does not eliminate the infection (7). The asymptomatic malaria cases were formerly common in high transmission settings in Sub Saharan Africa and some regions of Southeastern Asia but currently have also been reported in low transmission settings of South America as well (8). These infections may be associated with chronic and debilitating episodes of symptomatic parasitemia. Early detection and treatments of these cases is of paramount importance towards successful malaria elimination in endemic areas. *Plasmodium* spp. parasitemia involved in asymptomatic malaria infection is usually low and would thus require a high sensitive diagnostic tool (7, 6).

Microscopy and Rapid Diagnostic Tests (RDTs) which are the commonly used methods for malaria diagnosis because of their simplicity and inexpensiveness, have a challenge of low sensitivity (9-12). Molecular based techniques can overcome this challenge by not only being able to detect *Plasmodium* spp. in lower densities but are also capable of reliably identifying and differentiating between *Plasmodium* spp and mixed infections as well (10, 11, 13, 14). High resolution Melting (HRM) assay is a post PCR molecular method of genotyping which is attached to PCR amplification. HRM assay is a high sensitive, simple, rapid and accurate method based on the generation of melt curves and is very instrumental towards diagnosing *Plasmodium* spp. in low parasitemia based on a previously conducted study (10).

The aim of this study was to detect *Plasmodium* spp infections among non-febrile individuals living in a malaria endemic area of Southeastern Iran which is under intense elimination exercise using Real Time Polymerase Chain Reaction coupled with High Resolution Melting (Real Time PCR-HRM) analysis.

Materials and Methods

The study settings

This cross sectional study was conducted from 2016 to 2017. The study involved sample collection from Sistan and Baluchestan Province in Southeastern Iran and laboratory experimental implementation in Malaria Referral Laboratory of Tehran University of Medical Sciences.

Ethical consideration

The study was approved by the ethical committee of Tehran University of Medical Sciences (approval ref. No: IR.TUMS.VCR.REC. 1396.3314). The importance of the study was explained to the participants in their local language. Consent was obtained from all of the study participants. The Parents and the guardians consented on behalf of the study participants with age below 18 years.

Study sites

This study was conducted in Sarbaz and Chabahar Districts of Sistan and Baluchestan Province in southeast of Iran (Fig. 1). This province is the largest in Iran with an area of 181,785km^2 and a population of 2.775 million. The East of the Province borders malaria endemic countries of Afghanistan and Pakistan and Oman Sea to its South. The province accounts for one of the driest regions of Iran with a slight increase in rainfall from east to west, and an obvious rise in humidity in the coastal regions. It has hot and dry weather with about 65mm rainfall annually and accounts for the most malaria cases in Iran. About 92% of malaria cases in this region are caused by *P. vivax* and the balance by *P. falciparum.* In the recent years, *Anopheles stephensi* and *An. culicifacies* s.l. are the most important malaria transmission vectors in this region. The area experiences two malaria transmission peaks which are spring and autumn seasons (15, 16). The hospitals in these areas rely majorly on microscopy and RDTs for malaria diagnosis.

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Human blood sampling

The sample collection was done from 271 healthy individuals (171 native Iranians and 96 immigrants from Pakistan and Afghanistan) who were residing in the study areas for at least 6 months and had no any signs of febrile illness including fever, chills and rigor. The blood sample collection was done with strict adherence to the universal precautions for handling and disposal of human blood including: cleaning and puncturing the middle finger for preparation of thick and thin Giemsa stained blood slides according to the World Health Organization (WHO) malaria microscopy standard operating procedure (17). One blood spot from every study participant was put into a DNA banking card (Kawsar® Genomics, Iran) for molecular analysis. Another blood spot was put in RDT cassette (Premier Medical, India) and the results read immediately in the field in accordance to the WHO recommendations (18). The samples were then transferred by air to Tehran for molecular analysis. Patients who were perceived to have febrile illnesses resembling malaria infection were excluded from this study but were referred to the hospital.

Microscopy examination

Microscopic examination was done with strict adherence to World Health Organization (WHO) malaria microscopy standard operating procedure (17). The examination was done by two experienced microscopy experts who checked each duplicate of samples from each and every individual using X100 power objective power ocular lens. The slides were labelled negative if there were no any stage of *Plasmodium* spp seen after 100 microscopic fields were checked (17).

Molecular Analysis

Primer selection

A pair of primers sequence used in this work was selected based on a previous study by Chua et al. 2016 and was aimed at amplifying the target sequence located at 18s SSU rRNA gene of *Plasmodium* spp. The forward primer was 5'-GRAACTSSSAACGGCTCATT-3' and reverse primer 5'ACTCGATTGATACACACTA-3' (10).

DNA Extraction

DNA extraction was done based on a previous study by Bereczky using Tris-EDTA buffer (19) Tris-EDTA buffer contains 10mM Tris which is a mixture of Tris base and Tris HCl to a pH of 8.0; and distilled water dissolved 0.1mM EDTA. After the preparation, the Tris EDTA buffer stock solution was kept at a room temperature.

Punches from a DNA banking cards were cut into smaller pieces and put in a specific Eppendorf tube. 65µl of TE buffer was added to the Eppendorf tube containing the sample and vortexed. The samples were then put in a water bath at 50 ^{o}C for 15 minutes accompanied by gentle pressing with a fresh micropipette tips for every sample. The water bath temperature was adjusted to 95 ^{o}C with 15 minutes heating to elute the DNA from the samples. The samples were then centrifuged at 13,000 RPM for 1 minute to condense the liquid on the lid and the wall of the tubes. To confirm the concentration of the DNA, spectrophotometry (Nanodrop 2000®, Wilmington USA) was performed. The DNA extracts were then stored at -20 ^{o}C until was required to be used for HRM analysis (19).

The control templates

The positive controls were laboratory samples from *P. falciparum* and *P. vivax* infected human patients. These patients tested positive for both the microscopy and RDTs. For further scrutiny of the positive control, 30µl of their DNA templates was sent for sequencing (Macrogen, Korea). The nucleotide sequence was then compared with the sequences in the gene bank. The negative control was distilled water.

Reaction Mixture preparation

The HRM reaction mixture was made in a final volume of 20µl. The components includ-

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ed: 9µl of distilled water, 0.5µl of the forward primer (5'-GRAACTSSSAACGGCTCATT-3'), 0.5µl of the reverse primer (5'-ACTCGA TTGATACACACTA-3'), 5µl of EvaGreen® dye and 5µl of DNA template. The final volume of each sample was put in HRM strips and fixed with optical caps (Gunster Biotech, Taiwan) and loaded into the HRM machine (Step One plus ™) for analysis.

High Resolution Melting (HRM) analysis

The HRM analysis set conditions included primary denaturation at 95 ºC for 5 minutes, followed by 50 cycles each of: denaturation at 95 ºC for 25 seconds, annealing at 55 ºC for 30 seconds, extension at 72 ºC for 30 seconds and final extension at 72 ºC for 5 minutes. The HRM section included temperature increase from 65 ºC to 90 ºC with a temperature shift interval of 0.3 ºC.

To check for the consistency of the result, the positive clinical samples were repeated 4 times in different times but under the same condition. The HRM curves were generated and analyzed using High Resolution Melt Software v 3.0.1 (step one plus).

Results

Characteristics of study participants

The study participants were residents of Sistan and Baluchestan Province, southeastern of Iran. 64% (n= 175) of the study participants were Iranians and the balance (n= 96) were immigrants from Afghanistan and Pakistan. All of the study participants had no clinical symptoms of malaria or any other febrile disease that seems to resemble malaria paroxysm including fever, chills and rigor. 140 of the study participants were males and the rest (131) were females. About 46.4% of the males (n= 65) were native Iranians and the rest (n= 75) were immigrants. About 83.9% of the females (n= 110) were native Iranians and the balance (n= 21) were immigrants. None of the participants had either a history of malaria or has been on an antimalarial therapy within the past six months.

Sequence analysis for the positive control samples

A multiple sequence alignment between the nucleotide sequences of experimental positive controls of *P. falciparum* and *P. vivax* (Fig. 2) and the nucleotide sequences from the gene bank standards of *Plasmodium falciparum* 3D7 (CPO16995.1) and *Plasmodium vivax* sal-1 (UO3079.1) done using clustal W2 confirmed a close homology (99.44% and 90.37% respectively) between them as shown in the percentage identity matrix. The phylogenetic tree also confirmed a close relation (Fig. 3).

Plasmodium spp prevalence detection using the Rapid diagnostic tests (RDTs), Microscopy and High Resolution Melting analysis

Microscopy (through checking of 100 fields in thin blood films) and RDTs did not detect *Plasmodium* infection from 271 clinical samples (Table 1). The NanoDrop carried out confirmed a good concentration of DNA. The HRM revealed three infections from 271 clinical samples: two *P. vivax* infections and one *P. falciparum* infection.

High Resolution Melting analysis

The HRM analysis was done using HRM melt software v 3.0.1 (step one plus) (Fig. 4) Various forms of curves were generated including Amplification plots, derivative melt curves, and aligned melt curves for the three clinical positive *Plasmodium* spp. based on the two positive controls. The positive clinical samples were easily identified based on the nature of the curves that aligned with the positive control curves.

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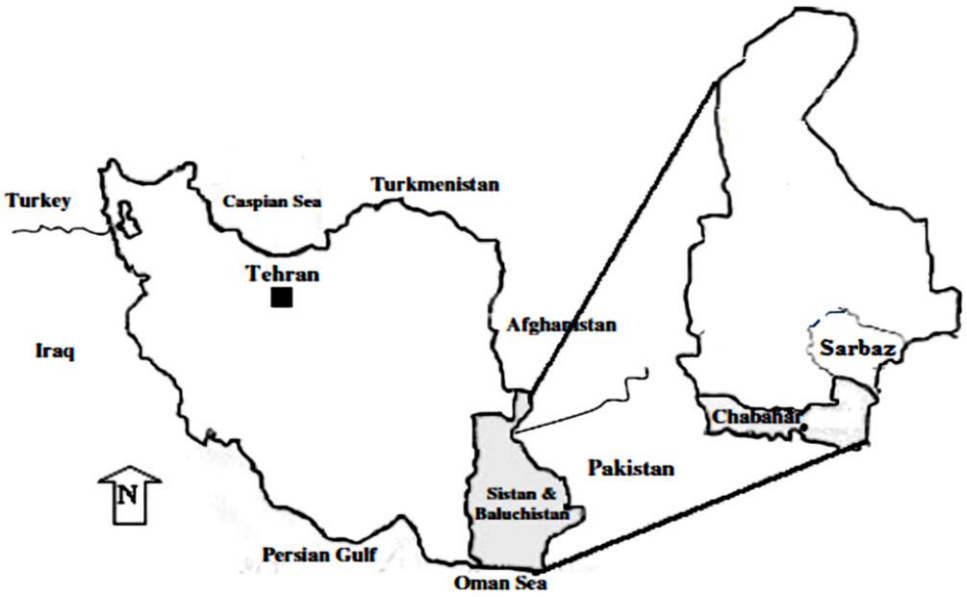

Fig. 1. Study area in Sistan and Baluchestan Province, Iran, (http://ircmj.com/168.fulltext)

A

>171016-044_A01_Pf1_spp_F.ab1 427
AAACTAAGCAACCCGACTACTTTATTATAAGGATAACTACGGAAAAGCTGTAGCTAATACTTGC
TTTATTATCCTTTGATTTCTATCTTTGGATAAGTATTTGTTAGGCCTTATAAGAAAAAAGTTATT
AACTTAAGGAATTATAACAAAGAAGTAACACGTAATAAATTTATTTTATTTAGTGTGTATCAAT
CGAGTAATGTGTATCAATCGAGTAGTGTGTATCACTCGAGTAGTGTGTATCAATCGAGTAGTGT
GTATCAATCGAGTAGTGTGTATCACTCGAGTAGTGTGTGTGCCTCCAAGATTGCGCATCACGCA
AGGCAAGAATGGGCCGCACACTCGGGTGGGCGGCATCCCGACCCGGACAACACACCCGGCCTC
CCCCCAATGGACCCCCAATAAAAAAATAAAAAAAAAAAAAAAA

B

>171016-044_C01_Pv1_spp_F.ab1 200
CAAAGGGCACTCGACTTTTTTCTATTAGGGTAACTACCGGAAAAGCTGTAGCTTATACTTGCTTT
AGCACTCTTGATTCATTTCTTGAGTGTGTACTTGTTAAGCCCTTTAAGAAAAAAGTTATTAACTT
AAGGAATTATAACAAAGAAATGACACGTAATGGATCCCTCCATTTTTATTGTGTATCAATCGAG
TAAAGT

Fig. 2. The nucleotide sequence for the experimental positive controls (A) *Plasmodium falciparum* (MG551534) and (B) *P. vivax (*MG551535)

Percent Identity Matrix - created by Clustal2.1

1: Pf1	100.00	99.44	76.70	83.52
2: P.f3D7	99.44	100.00	76.14	82.95
3: Pv1	76.70	76.14	100.00	90.37
4: P.vivaxSal1	83.52	82.95	90.37	100.00

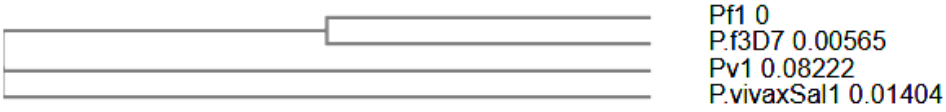

Fig. 3. The homology between the *Plasmodium falciparum* and *P. vivax* experimental positive control samples and the *P. falciparum* 3D7 (CPO16995.1) and *P. vivax* sal-1 (UO3079.1) gene bank standards nucleotide sequences. (A) Percentage identity matrix and (B) phylogenetic tree. Both the identity matrix and the phylogenetic tree shows a close identity between the experimental positive controls and the standards from the gene bank

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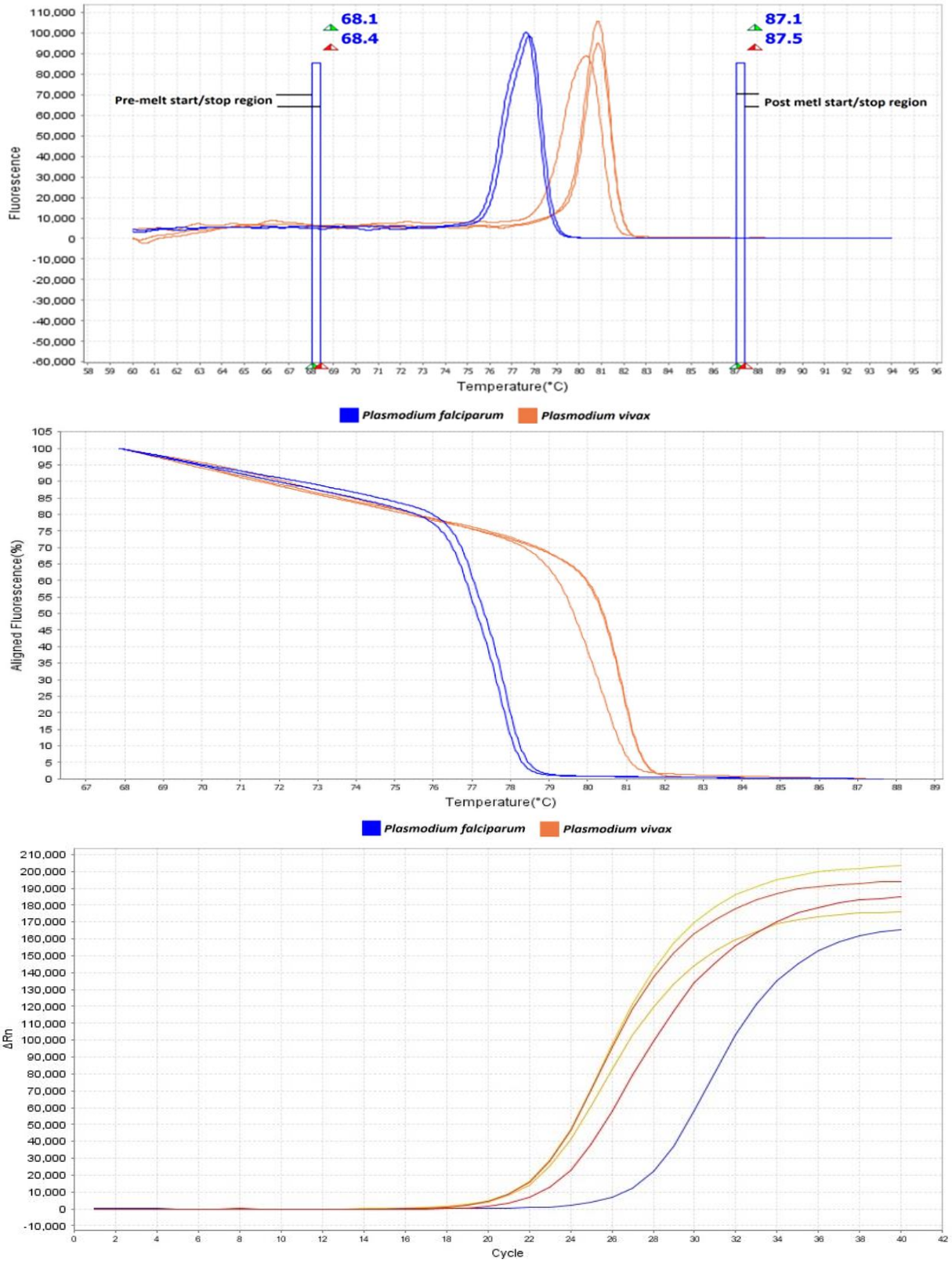

Fig. 4. Melt curves of the positive clinical samples and the experimental positive controls (A) the amplification plot, (B) derivative melt curves and (C) aligned melt curve

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Table 1. Asymptomatic malaria cases detection results based on Rapid diagnostic tests (RDTs), Microscopy and High Resolution Melting assays

Nationality	Number of Study Participants	Number of Microscopy Positive Cases	Number of RDTs Positive cases	Number of HRM Positive Cases	
Iranian	175	0	0	Male	1
				Female	1
Immigrants	96	0	0	Male	1
				Female	0
Total	271	0	0	3	

Discussion

The curves for the three clinical *Plasmodium* spp positive samples aligned to the curves of the positive control samples consistently throughout the four trials. 18S SSU rRNA was selected for use in this study based on the previously molecular studies. This region is much conserved and highly variable having multiple copies which are scattered throughout the genome of *Plasmodium* spp making it suitable for molecular amplification (10, 20).

Upon the sequencing of the positive control samples for this study, the nucleotide sequence of the positive controls used (*P. falciparum* and *P. vivax*) showed a close homology with the gene bank reference sequences for *P. falciparum* 3D7 (CPO16995.1) and *P. vivax* Sal-1(UO3079.1).

The principals behind HRM analysis towards detections and differentiation of *Plasmodium* spp is based on the nature of melt curves and melting temperature (Tm). The HRM assay set up is the same as with the RT-PCR procedure with a difference of incorporation of a fluorescent intercalating dye in HRM that is used to monitor the transition of DNA from double stranded (dsDNA) to single stranded (ssDNA) and assigning the Tm in the process. Tm is a point in which dsDNA is equal to ssDNA, this point is very significant towards species differentiation and *Plasmodium* spp mixed infections detection (21, 22).

In this study, three individuals out of 271 non-febrile study participants (1.1 %) from the malaria zone of Iran have been found to be infected with *Plasmodium* spp. This is in a close conformity with other molecular based asymptomatic malaria surveillance findings from the same malaria endemic areas of Iran: Amirshakeri et al. and also Turki et al. reported 0.33% and 1.5% asymptomatic malaria infections respectively from the surveys they conducted (22, 23). The parasitemia involved in these infections could neither be detected by microscopy nor RDTs. However in another microscopy based asymptomatic malaria prevalence survey that has been conducted by Nateghpour et al. in Iranshahr District of Sistan and Baluchestan Province, neighboring the studied districts, they reported 1.6% and 0.6% *Plasmodium* spp prevalence among Afghanistan immigrants and native Iranians respectively (24). Obviously, according to WHO guidelines the number of parasites per microliter of blood is crucial for detecting them either with microscope or RDTs. The density of parasite is not less than ten and forty for microscopy and RDTs respectively. So, discrepancies among results of the mentioned references may be interpreted due to the above mentioned reason. These infections if left undetected and untreated would act as reservoirs and play a crucial role towards the transmission of malaria within a population where they are prevalent and would thus consequently delay or prevent malaria control and elimination exercises in malaria endemic areas. On the other hand, some

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molecular based studies conducted in the south-eastern malarious areas of Iran resulted in absence of asymptomatic malaria (25, 26).

Kipanga and colleagues were able to diagnose and detect *Plasmodium* spp from microscopically negative febrile patients by using PCR coupled with HRM from hospitals in Kenya who could not be put on antimalarial drugs (12). Chua et al. identified *Plasmodium* spp in 11 out of 14 microscopically negative febrile patients using RT-PCR coupled with HRM (10).

Iran has achieved tremendous successes towards the elimination of malaria by using methods like: passive and active case detection, vector control methods, and effective treatment using artemisinin combination therapy (ACT). These strategies have reduced malaria infections in the country from 12,294 to 1,251 between 2000 and 2014 (22-24). Asymptomatic malaria may however rekindle malaria infections or slow down the pace of the elimination programs being undertaken.

Apart from the aforesaid, HRM assay has also been successfully used in other parasitological studies including: identification of mutant strains of *P. falciparum* that are drug resistant; diagnosing the genetic diversity of *Dientamoeba fragilis* isolates of irritable bowel syndrome patients, analyzing the single nucleotide polymorphism of *Echinococcus granulosus*, detection and identification of Old World *Leishmania*, detection and identification of Human Hookworm infections. These studies have demonstrated a high sensitivity nature of HRM in diagnosis compared to other parasitological methods (27-31). Although HRM assay is very sensitive in diagnostics, its initial cost and operational costs are more expensive than microscopy and RDTs.

Conclusion

In conclusion HRM assay is a favorable method towards the rapid detection of malaria in low parasitemia below the LoD of microscopy and RDTs and it is therefore important in areas where malaria elimination exercises are being carried out particularly if the malaria policy makers want to remove most hidden obstacles including asymptomatic malaria from their pathway. This method is also capable of differentiating between *Plasmodium* spp. by just using a single pair of primer sequence making it simpler to use compared to other diagnostic methods. Coming up with this kind of an assay which would incorporate DNA extraction from the samples prior to their amplifications would be a great development as it would avoid the errors associated with the DNA extractions that may affect the results of HRM assay.

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The authors declare that they have no competing interests.

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