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Original Article

Simplified Pan-species Real-time PCR-based Detection of *Plasmodium* Spp. in Blood Smear

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Abstract

Background: We aimed to quicken and simplify the detection of *Plasmodium* in blood samples by developing and testing a pan-*Plasmodium* real-time PCR for accurate screening of individuals suspected of malaria.

Methods: A single primer/probe set for pan-species *Plasmodium*-specific real time PCR targeting a conserved region of the small subunit 18S ribosomal DNA was designed and evaluated for rapid diagnosis and screening of malaria infections using dried blood smears. FTA cards were used for rapid and simple DNA extraction.

Results: The primers and probes showed a positive response with the DNA extracted from bloods infected with *P. falciparum* and *P. vivax* but not with DNA extracted from various smears from uninfected blood samples. Seven positive cases positive by both microscopy and nested PCR were found among 280 blood samples taken from in South and Southeast Iran. Five samples were identified as positive for *P. vivax* and two as positive for *P. falciparum*. All positive samples were positive by real-time PCR. Furthermore, all 38-blood samples positive by microscopy were positive by real-time PCR. No microscopy-negative samples were positive by real-time PCR.

Conclusion: By using a simple FTA card for DNA extraction and by application of the real-time PCR developed in this study, sensitivity similar to nested-PCR and microscopy was achieved. This format simplifies the detection of *Plasmodium* in large numbers of samples.

Introduction

Despite considerable efforts, malaria is still one of the most overwhelming infectious diseases and endemic in some parts of the world. The disease is widespread in the tropical and subtropical regions making up a wide-ranging band around the equator (1). Of the approximately 3.4 billion people worldwide who are exposed annually, 1.2 billion are at high risk. World Health Organization (WHO) states that in 2015, 96 countries and territories had ongoing malaria transmission with an estimated 438,000 deaths (2). Malaria is commonly associated with poverty and has a major negative effect on economic development (3, 4).

Iran's efforts to control malaria over the recent decades have been largely successful; however, a limited number of cases have been found in the southern and eastern parts of the country (5, 6). These cases are linked to the migration of immigrants. Therefore, finding and tracking infected cases is critical to identifying transmission route, controlling planning, and eventually interrupting the transmission cycle. This action, however, is impeded by the fact that many blood samples should be subjected to diagnostic testing in order to discover the small number of cases. Moreover, cases with low parasitemia challenge the sensitivity of available diagnostic methods (7). For this reason, it is necessary to increase our capacity to identify cases and develop a relevant treatment system. Standard diagnosis still relies on traditional microscopy of blood smears. The quality of smears, including the staining, the quality of microscope, and, most importantly, the skills of the microscopist affect the results of microscopy. Elimination programs require more sensitive methods to not only reduce the risk of generating false negative results as much as possible, but also to be able to carry out massive screening of a large number of specimens.

Over the recent decades, molecular techniques including PCR have been successfully used to detect malaria infection and/or identify its causative agents (8), and such methods are continuously being developed. Several studies have used PCR and real-time PCR variants, mostly involving commercial kits (9-11). These kits are largely too time-consuming to use and often too expensive for laboratories in developing countries (12) There are also reports on multiplex real-time fluorescent-based or probe-based PCR methods aiming to both detect and identify *Plasmodium* species in blood samples. However, takin into account the limited number of malaria cases in those countries that are in elimination phase, the main goal at the first step should be a cost-effective screening of a large number of samples to identify the limited number of cases; the identification of the causative species can then be performed by complementary steps.

In this study, we aimed to quicken and simplify the detection of *Plasmodium* in blood samples by developing and testing a pan-*Plasmodium* real-time PCR for accurate screening of individuals suspected of malaria.

Materials and Methods

Samples

Three groups of samples were used in this study: 1) Blood samples taken from a patient infected with *P. falciparum* for preparing serial dilutions of DNA to determine the sensitivity of both extraction and PCR methods; 2) a total of 280 blood samples from the southern and southeastern parts of Iran, examined by light microscope, nested PCR and real-time PCR; and 3) a total of 38 blood samples collected over the past few years that were positive by direct microscopy and identified as *P. falciparum* or *P. vivax*. Blood samples from the first and second groups were each smeared onto at least two slides; one for staining and microscopy and another without fixing and staining for DNA extraction and molecular tests. With regard to the third group, frozen

blood samples were directly applied to FTA cards for DNA extraction and PCR.

The study was approved by Ethics Committee of Tehran University of Medical Sciences.

Development of a real-time PCR assay for detection of Plasmodium spp. in blood

After *in silico* analysis of several sequences copied from GenBank, in the genus of *Plasmodium,* 18S small subunit ribosomal DNA (SSU rDNA) showed conserved regions across species. Therefore, this genomic part was selected as the target for universal detection of *Plasmodium* spp. To select the appropriate probe and primers, various sequences of SSU rDNA related to different *Plasmodium* species were retrieved from GenBank and subjected to multiple alignments by the software Geneious 5.6.5 (http://www.geneious.com) and BioEdit (http://www.mbio.ncsu.edu). According to the conserved and variable regions in the aligned SSU sequences reflecting various species of *Plasmodium*, and considering the upstream and downstream sequences of the same region in the corresponding human gene (Fig. 1), a pan-*Plasmodium* primer/probe set was designed using Genscript software (https://www.genscript.com). The probe was synthetized by Bioneer (South Korea) and labeled with FAM at the 5´-end as the reporter dye and TAMRA at the 3´-end as the quencher.

Real-time PCR

Each reaction contained 3 µL of the extracted DNA, 0.5 µL (0.3 mM) of each primer, 0.25 µL (0.15 mM) of the probe, 7.5 µL of premix (Takara, Japan), and 3.25 µL of distilled water in a 15-µL reaction volume. PCR was performed using 0.1-mL tubes or 48-well plates with the following program: One-step of 95 °C for 3 min followed by 40 cycles each comprising a step of 95 °C for 5 s and 60 °C for 20 s. Data were collected at the end of the final 60 °C stage. PCR was performed using a StepOne machine (Applied Biosystems, Singapore), and data were analyzed using the StepOne software v2.3.

Real-time PCR sensitivity and specificity

In order to evaluate the limit of detection of the PCR, 50 µL distilled water was added to an unstained *P. falciparum*-positive blood smear. After scraping the blood film off the slide, the suspension was divided into two parts: One portion was submitted to DNA extraction using a commercial DNA extraction kit (GenAll, Korea), for which the protocol was followed as recommended by manufacturer, and the DNA was eluted in 30 µL distilled water. Moreover, five-µL aliquots of the suspension were added to a 3-millimeter-in-diameter-punched FTA classic card (Wathman, USA) and left to dry for at least 3 h at room temperature. For DNA extraction, each card disc was added to a tube containing 500 µL distilled water, and after a short vortex, was added to 0.2-mL tubes containing 30 µL distilled water and incubated at 95 °C for 20 min, after which the disc was removed and DNA was stored in -20 °C until use. Both types of DNA extracted (extracted by the commercial kit and FTA cards, respectively) were submitted to twice-serial dilution so that dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 of each DNA sample was obtained. Three microliters of each dilution were used per PCR reaction, and the experiments were repeated at least 3 times.

Nested PCR

Since nested PCR has recently been introduced as the gold standard and the most sensitive method for detection of *Plasmodium* in blood samples (21), nested PCR was compared with real-time PCR in terms of sensitivity. For nested PCR, 3 µL of template DNA, 0.5 µM of each primer (14), 12.5 µL premix (Ampliqon, Denmark), and enough distilled water were mixed to make up a 25-µL reaction volume. The two-step nested PCR was performed as described previously (14). PCR products were electrophoresed on 2% agarose

gels for one hour in TBE buffer (Tris 90 mM, Boric-Acid 90 mM, EDTA 2 mM), stained with 0.5 µg/mL ethidium bromide, and visualized under a transilluminator.

In all PCR reactions, two positive controls (DNA extracted from blood samples positive with *P. falciparum* and with *P. vivax*), and at least two negative control samples containing water instead of DNA, were included in each PCR. For samples with false negative or false positive results, the PCR was repeated.

Results

The selected oligonucleotides for the probe-based real-time PCR were as follows: 5'-AGCTCTTTCTTGATTTCTTGG-3' as the forward primer, 5'-CAGACAAATCATATTCACGAACT-3' as the reverse primer, and AAAC-GGCCATGCATCACCAT as the TaqMan probe (reverse direction). Fig. 1 shows the results of multiple alignment of representative partial 18S rDNA sequences reflecting different *Plasmodium* species and the position of the selected primers and probe. The target is a 66-bp long conserved sequence fragment that matches all species within the *Plasmodium* genus, including *P. falciparum* and *P. vivax*, which are the most common species identified in Iran and many other countries, and excluding human DNA. Reconfirmation of the oligonucleotides was done by BLASTing the oligonucleotides to the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov).

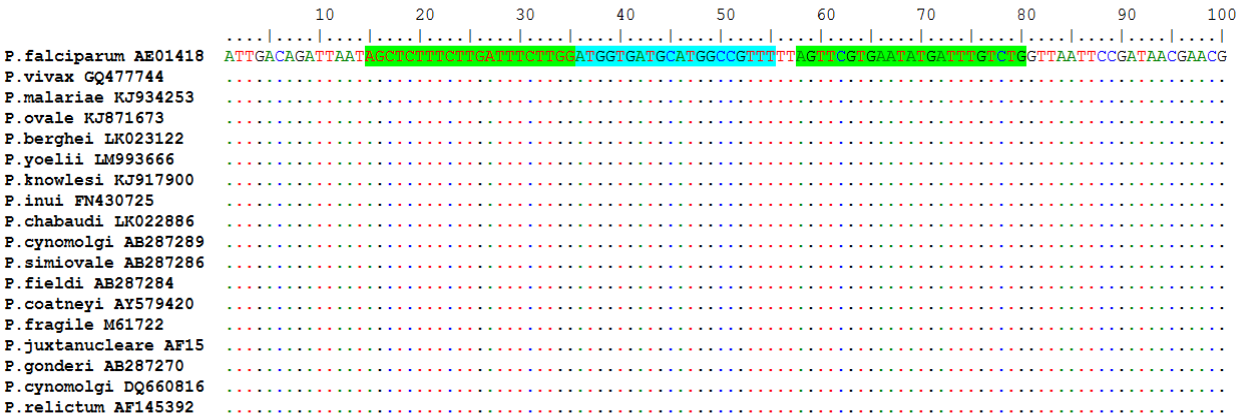

Fig. 1: Multiple sequence alignment of partial small subunit ribosomal DNA (18S rDNA) sequences of representative *Plasmodium* species. A dot indicates an identical nucleotide with respect to the top sequence. The position of the forward and reverse primers as well as the TaqMan probe is marked. Note that the probe described in the text was indicated in the reverse direction

In practice, the primers and probes amplified and detected *Plasmodium*-specific DNA extracted from the blood samples infected known to be positive for *P. falciparum* or *P. vivax*, while no cases of false-positive cases were seen with template DNA extracted from various smears of non-infected blood samples.

To determine the analytical sensitivity of the real-time PCR assay, PCR was carried out with the serially diluted DNA extracted using the commercial kit and FTA cards. An excerpt of the results is given in Fig. 2, A. Real-time PCR enabled successful amplification and detection of *Plasmodium*-specific DNA extracted by FTA cards and produced amplification plots for the dilutions 1:1, 1:2, 1:4, and 1:8. Using the commercial kit, the dilution of 1:16 was positive only on some occasions, and, surprisingly, the undiluted samples (1:1) were always negative.

A total of 280 blood samples taken from the southern and southeastern part of the country,

including samples from Pakistani and Afghani immigrants, were tested in this study. Two slides were prepared from each case; one was observed by light microscopy upon staining, and the other slide was used for DNA extraction using the FTA card method and examined by both nested PCR and real-time PCR (Fig. 2, B).

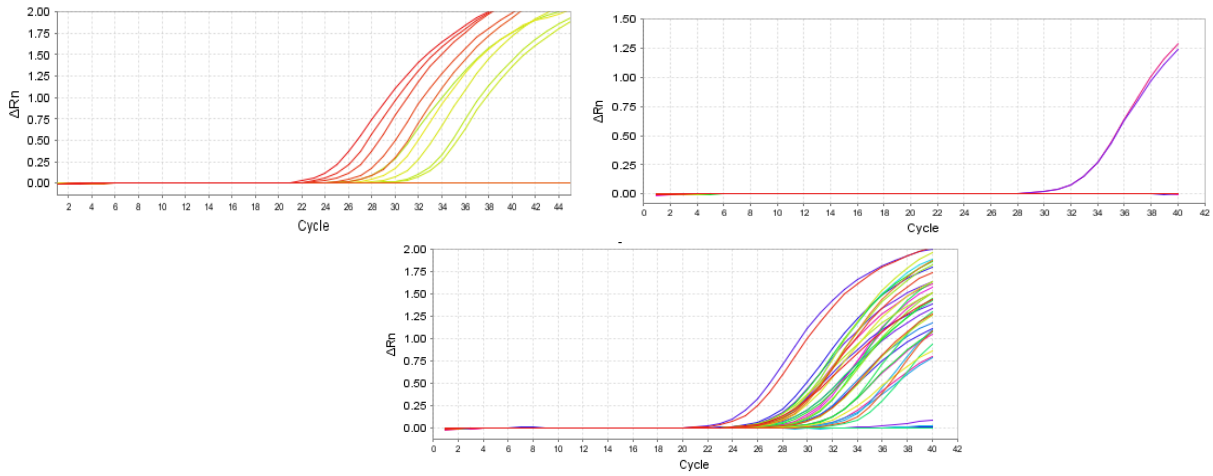

Fig. 2: Amplification plot (ΔRn *vs.* cycle) showing real-time PCR-based detection of *Plasmodium* species in human blood samples. A) Serial dilutions of DNA extracted by a commercial kit are indicated by red lines, while templates DNA from FTA cards are indicated by green lines. B) An example of real-time PCR performed in duplex for an individual suspected of malaria infection. C) Thirty-eight microscopy-positive blood samples were tested with real time PCR

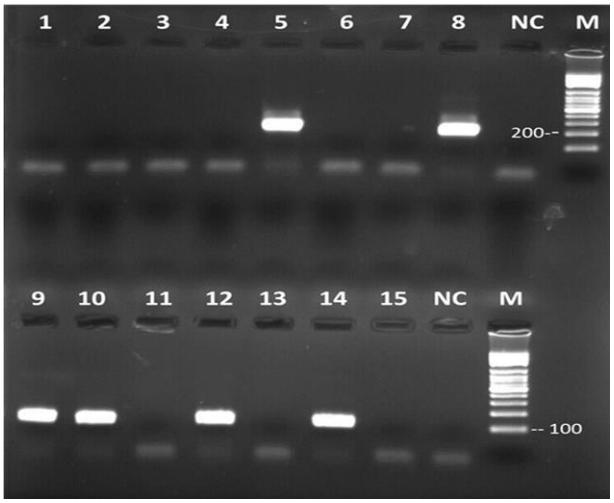

Fig. 3: Agarose gel electrophoresis of the products obtained by nested PCR of DNA extracted from some blood samples. Lane 5: a positive control of *P. falciparum*; lane 8: a positive sample of *P. falciparum*; lane 9: a positive control of *P. vivax*; lanes 10, 12, and 14: samples positive for *P. vivax*; lane NC: negative controls; lane M: 100-bp DNA size marker

Seven positive cases were positive by both microscopy and nested PCR, by which, according to the band size, five were identified as *P. vivax* and two as *P. falciparum* (Fig. 3). No samples negative by direct microscopy were positive by nested PCR. All positive samples were also found to be positive by real-time PCR. Furthermore, all 38-blood samples positive by microscopy were positive by real-time PCR (Fig. 2, C). Thus, it is observed that by using a simple FTA card for DNA extraction and by application of the real-time PCR designed in this study, sensitivity similar to that of nested PCR and microscopy can be achieved, at least for the samples tested in this study.

Discussion

Malaria is one of the most important infectious diseases in tropical areas with an added risk of imported cases to all non-endemic countries with conditions conducive to disease transmission. Malaria is still a lethal infection

worldwide, responsible for approximately 0.5 million deaths annually, particularly in children (2,15). The disease is endemic in many countries, causing significant mortality and morbidity and posing economic burdens (16). While the number of cases of malaria in counties where the disease is under control is decreasing, imported malaria is an important emerging disease (17, 18). One reason for this is the increasing movement of immigrants to other countries (8). Iran has a program for eliminating malaria, and this program aims to have reduced local transmission cases to zero by 2025 (2).Population movements and altered transmission patterns through changes in populations, culture, and even climate are risks that should be considered in every comprehensive eradication program. Many legal/illegal immigrants enter the country every day. This population is a potential obstacle for elimination programs to reach success, and as immigrants move to the inner parts of the country, the problems could be transferred to new areas. Another problem pertains to carriers with low parasitemia, which is difficult to detect by conventional diagnostic tools. Five *Plasmodium* species can cause disease in human; including *P. vivax* and *P. falciparum*, which are the most frequent species encountered in Iran and neighboring countries.

Timely diagnosis of the disease is a precondition for any control program. The least expensive method for diagnosing malaria is undoubtedly microscopic examination of thick and thin blood smears. However, several issues are linked to this traditional approach: 1) the sensitivity of direct microscopy is dependent on the skills of the microscopist; 2) in areas where malaria is not endemic, maintaining a sufficient level of technical expertise in malaria identification is costly (19); 3) examination of a large number of samples to identify the limited number of positive cases is time-consuming and labor-intensive, and 4) low-parasitemia cases may be missed during microscopy-based massive screening.

Recently, molecular diagnostic tests, in particular real-time PCR, have been introduced for the detection of *Plasmodium* parasites in blood samples, and apart from high sensitivity, such assays are automatable, standardizable, and quantitative. A variety of real-time PCR methods for malaria have been described and evaluated as sensitive and specific (8). The reported methods have advantages such as sensitive detection as well as species identification of *Plasmodium* spp., including rare species such as *P. ovale*, *P. malariae*, and *P. knowlesi* .

In the present study, we introduced a pan-species *Plasmodium*-specific real-time PCR for the diagnosis of malaria infections. Prior to developing such the PCR, the following points were considered: 1) Instead of using fresh blood samples that usually necessitate invasive sampling and require facilities such as refrigerator to store the specimens, dried blood samples on a slide (smears) could be used for DNA extraction followed by PCR amplification. Therefore, the collected samples can be used not only for microscopy, but may also be archived for subsequent molecular tests. Although stained smears were not used in this study, slides that have been stained can first be used for microscopy and then for molecular testing. Alternatively, the two halves of one smear may be applied for direct microscopy and PCR, respectively. 2) Instead of using conventional methods or commercial kits that are usually expensive for DNA extraction, FTA cards can be used for rapid and simple extraction of DNA. The cards have already been used and evaluated as a valid tool for DNA extraction from many types of samples, including blood (20). FTA cards also represent an excellent tool for archiving nucleic acids and keeping the samples for further tests or researches. 3) Instead of using several primer/probe sets to detect/identify specific species, which is not only more expensive but also requires setting up a multiplex PCR using a multichannel PCR machine, a single primer/probe set was designed to enable pan-*Plasmodium* detection of all species. As the tar-

get of the primers and probe, we used 18S ribosomal DNA, which is a well-described target. This gene has already been used in several reports as a good marker for *Plasmodium* spp. as well as many other pathogenic parasitic or fungal eukaryotes. This simplified format makes it possible to detect straightforwardly any *Plasmodium* in a large number of the samples. Probably (although rare) positive cases can be cumulatively subjected to molecular characterization (ideally by PCR and sequencing) to identify the species of *Plasmodium* for any therapeutic or epidemiological purposes. In this study, we had seven positive samples that were easily identified by the already described nested-PCR format (14).

In practice, our set-up worked well in the way that in comparison with direct microscopy, which is the method considered having best specificity, and with nested-PCR, which can be considered the gold standard regarding sensitivity, our simple real-time PCR had similar or better sensitivity and identical specificity. Among the 280 blood smears tested, a total of 6, 6, and 7 samples were positive using microscopy, nested-PCR, and real time PCR, respectively. Furthermore, all 38 blood samples, which were positive by microscopy, were also positive by real-time PCR after application of FTA card as the DNA isolation tool. These results might validate the method in term of sensitivity and specificity.

Conclusion

By using a simple FTA card for DNA extraction and by application of the real-time PCR assay designed in this study, sensitivity similar to nested PCR and microscopy was achieved. This format simplifies the detection of any species of *Plasmodium* in a large number of the samples. Although the sensitivity of using DNA extracted with the FTA card was compared with commercial kits via preparation of serial dilution, however, for an exact evaluation of analytical sensitivity and specificity, calculation of parasite cells and/or cloning of the PCR target inside a plasmid and calculation of copy number are recommended.

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