

Detection of *Plasmodium falciparum* Directly from Blood Samples Using the Polymerase Chain Reaction

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Abstract

Malaria remains one of the major causes of human morbidity and mortality, particularly in some developing countries such as Iran. The importation of malaria also into a place or region where they are not endemic raises many concerns, including the timely delivery of appropriate care, safety of the blood supply, the risk of autochthonous and transmission. Although different methods are applicable for detection of parasites which cause malaria, some of them do not have enough sensitivity. In this study a direct polymerase chain reaction (PCR) and light microscopy approaches were compared to detect *Plasmodium falciparum* in terms of sensitivity and specificity using blood samples from people with malaria-like symptoms and fever. A target DNA sequence of the plasmodial 18S ribosomal DNA (206 bp) was amplified by PCR. The assay showed a threshold of parasite density (detection limit) of one *P. falciparum* parasite/20microliter. Matching results were obtained for 18 people who presented malaria-like symptoms and fever. Our results showed that the direct PCR method proved to be a more sensitive and useful tool than the light microscopy assay to determine *P. falciparum*. Microscopy assay showed a low sensitivity (37.3%) when compared with *falciparum*-DNA detection by direct PCR (87.5%). In conclusion it was determined that direct PCR is a reliable method for detection of malaria parasites and it may be a useful tool for detection of other parasites as well.

Keywords: Development; Malaria parasite; *Plasmodium falciparum*; Direct PCR; Iran

Introduction

Plasmodium falciparum causes the most severe form of human malaria. The World Health Organization (WHO) has estimated that between 300 and 500 million new clinical cases and as many as 2.7 million deaths

occur each year due to this infection [10]. It has been shown that malaria situation in south-eastern Iran is quite serious, due to its neighbor, Afghanistan; a country with disrupted health systems. This area is situated in the vicinity of Zahedan, the capital city of Sistan and Bluchistan province in the south-eastern part

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of the country, a typical zone of Iranian Bluch. During the humid and warm period of the year, from June to October, malaria transmission in this region is high, seasonal and peaking in September. Sabatinelli (1999) studied the malaria situation in the eastern Mediterranean region of WHO and reported more than 32,616 cases in 1998. An Annual Parasite Incidence (API) of 8.74 per 1000 populations has been reported for this area [7].

The extreme pathogenicity of *P. falciparum* seems to be related to the ability which causes infected red blood cells to adhere into vascular endothelium and sequester in the microvasculature of different organs [3]. Rapid and prompt diagnosis of malaria infections followed by adequate chemotherapy is a major tool in malaria control. Although a wide variety of methods have been used, traditionally, laboratory for detecting *Plasmodium* infection of malaria is based on Giemsa-stained blood smear. Even though successful and inexpensive, this method is backbreaking and time-consuming, and its sensitivity drops with the decrease of parasitemia [1,4,11].

Recently, other diagnostic methods, such as PCR, have been employed for the detection of malaria parasites. The PCR method effectively detects parasites in mixed and low level infections, being more sensitive when compared to microscopic examination [9] which validates its use in malaria epidemiological studies.

It is well understood that the success of the PCR technique depends on a range of factors such as high quality DNA obtained from blood samples, good reagents and sufficient conditions of amplification. Whole blood has been indicated to be a reliable source of high-quality DNA [6] when it is carefully transported, handled and stored to prevent contamination or degradation of the DNA.

In this study we conducted a simple method for treating blood samples permitting direct detection of *P. falciparum* parasites using amplification of *P. falciparum*-specific 18S rDNA sequences by PCR amplification of target DNA, and compared the results from this method with light microscopic examination of thick blood smears. The direct PCR results showed the remarkable sensitivity and specificity in comparison with microscopic examination. This method is suitable for detection of parasite carriers and evaluation of medical treatment on clinical cases.

Material and Method

Study Area

The study area was situated in the vicinity of

Zahedan, the capital city of Sistan and Bluchistan province in the south-eastern part of the country, a typical zone of Iranian Bluch.

Microscopic Examination of Blood Smears

In this study patients who were referring to the Central laboratory of Iranshar were recruited for malaria diagnosis. Blood samples were collected during the high transmission seasons (August-October) from 204 randomly selected asymptomatic individuals. Five milliliters of venous blood from each person were added to tubes containing EDTA. Furthermore, peripheral venous blood was used to prepare thin, thick blood films and PCR as well. Blood films were prepared from a droplet of the blood on a glass slide. Thin and thick films were stained with 10% Giemsa and examined and also re-examined by two technicians in the Central Laboratory of Iranshar. The parasitemia per microliter of blood was measured as parasite count = (white blood cell count × parasites measured per 100 white blood cells)/100. If a specimen was determined to be positive for *P. falciparum* parasites by the laboratory, the sample was selected for optimization of direct PCR confirmation.

Preparation of DNA Template

The isolation of DNA from blood was carried out as described by Tirasophon *et al.* (1991). Briefly, 20 µl of whole blood containing EDTA was poured into a 1.5-ml tube and lysed by adding 200 µl of R buffer (0.015% Saponin, 50 mM NaCl, 1 mM EDTA) after vortexing the samples were centrifuged for 10 min at 10,000 rpm. The supernatant was removed; 250 µl of R buffer added and centrifuged at the same condition. The pellets at this stage consisting human and malaria parasite DNA, were ready for PCR [12].

Direct PCR Assay for Parasite

Polymerase chain reaction (PCR) for amplifying a fragment of 18S rDNA containing repetitive sequences was carried out as described before [12]. Briefly, 49.5 µl of PCR mixture including 5x PCR buffer, 20 pmole of primers, MF (5'-CGCTACATATGCTAGTTGCCACAG-3') and MR (CGTGTACCATACATCCTACCAAC), 2 µl dNTP and 75 µl Mineral Oil were added to each sample, vortexed and boiled for 10 min. Subsequently PCR was conducted after addition of 0.1 u *Taq* polymerase PCR was carried out under the following conditions: 94°C for two minutes, 35 cycles at 94°C for 30 s and at 54°C for one minute, followed by a final extension at 72°C for 10 min. Subsequently, PCR fragments were resolved in 1.2% agarose gel, and stained with ethidium bromide for visual detection by ultraviolet transillumination [9].

Results

Prior to performing direct PCR technique 20 samples from individuals with malaria-like symptoms and fever with high *Plasmodium falciparum* parasitemia which had been detected by microscopic examination selected for optimization of direct PCR analysis. The assay is based on nucleotide primers designed to amplify a 206-bp fragment of ribosomal RNA (rRNA) subunit coding sequence within the 753 bp of DNA malaria genome.

Figure.1 presents gel electrophoresis of the 206 pb PCR products from positive controls with *Plasmodium falciparum*.

In order to determine the specificity of direct PCR, one sample which had been already detected with *plasmodium vivax* parasites was examined as described in Figure 1. The results from negative control and sample containing *Plasmodium vivax* confirmed the specificity of primer and PCR condition. Due to lack of pure DNA from *Parasitemia falciparum*, the sensitivity of the direct PCR was determined by the lowest *Parasitemia falciparum* using blood samples detected by light microscopy. Blood sample with low density of parasite was diluted in different ratios (Fig. 1, lane 2).

Subsequently, 18 samples from individuals who presented malaria symptoms were studied by the direct PCR. Although conventional examination detected six samples for *Plasmodium falciparum* and two samples for *vivax*, this technique was unsuccessful in the detection of 8 samples containing *Plasmodium falciparum*. Therefore 14 out of 18 samples with malaria symptoms were detected by the direct PCR.

Discussion

To date, light microscopic examination is still considered to be the most practical and reliable means to detect malarial parasites in blood samples [14]. It is inexpensive and can be employed under practically any field conditions. However, it is time-consuming and requires experienced staff. In addition, its sensitivity in identifying low numbers of parasites in the sample is limited [5]. To work out this limitation, PCR amplification of *Plasmodium* DNA in blood has been used as a highly sensitive tool for the diagnosis of malaria parasites. Furthermore, direct PCR can also be used for the detection of *Plasmodium* parasite without any DNA extraction. [12].

In choosing this approach there was an acute awareness of the abundant literature demonstrating that direct PCR has ability to detect parasites in the samples with lower degree of parasitemia. Direct PCR also offers several key advantages over difference-based

methods such as microscopic examination, ELISA and routine PCR which have inherent limitations. First and foremost, direct PCR has a major advantage in detecting direct *P. falciparum* and eliminates the requirement for DNA extraction and a specific antibody for protein, respectively.

In this study, we performed and evaluated a direct PCR method that could rapidly detect *Plasmodium* DNA in blood samples from people who admitted to Central Laboratory of Iranshar with suspected malaria. A 206bp of 18S rDNA sequences was amplified by PCR This parasite has four copies of the small (18S) rRNA subunit genes in the genome. Many studies have shown no significant homology among different malaria species and human for 18S rDNA of *falciparum*. We initially optimized the direct PCR protocol to detect and distinguish *P. falciparum* using different positive controls which were already diagnosed by light microscopy. Microscopic examinations demonstrated a low sensitivity (37.3%) when compared with direct PCR (87.5%). In conclusion it was determined that direct PCR method is a reliable method for detection of malaria parasites and it may be a useful tool for detection of others parasites. As well as present study, a number of previous studies have reported better sensitivity and specificity using direct PCR for the detection of the malarial parasite [15].

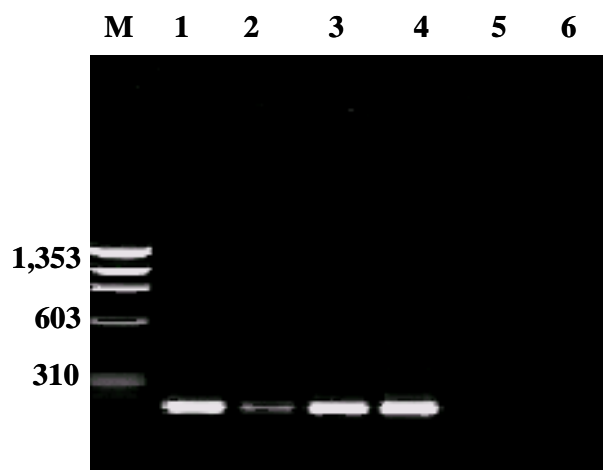


Figure 1. PCR amplification of 18S rDNA from of *P. falciparum*. 1.2% ethidium bromide stained agarose gel electrophoresis showing the molecular weight marker (PHIX174 Hae III), a 206 bp sequence of the small (18S) rRNA subunit genes of *P. falciparum* (lanes 1-4). Lane 2 indicates the sensitivity of direct PCR using the lowest parasitemia sample. Lanes 5 and 6 show the specificity of primers and PCR conditions using *Plasmodium vivax* and H₂O, respectively.

Consequently, these results from the direct PCR suggest that, in malaria endemic areas such as some part of Iran, the direct detection of malaria parasites by PCR can be a useful complementary tool to microscopic examination in order to detect each species of parasites and also for the follow-up of patients after specific treatment.

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