

Evaluation of Negative Malaria Giemsa-Stained Smears Referred from Malaria Centers in Sistan and Balouchestan Using Nested Polymerase Chain Reaction

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| Article information | Abstract |
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| <p>Article history: Received: 17 Dec 2012 Accepted: 26 Dec 2012 Available online: 26 Feb 2013 ZJRMS 2014; 16(4): 15-18</p> <p>Keywords: Nested polymerase chain reaction Negative smears Microscopic examination method</p> <p>*Corresponding author at: Department of Mycology and Parasitology, Tropical and Infectious Diseases Research Center, Zahedan University of Medical Sciences, Zahedan, Iran E-mail: Ebrahimzadeh@zaums.ac.ir</p> | <p>Background: Malaria is one of the most important infectious diseases worldwide and also in Iran. Reports announced by Controlling Diseases Center (CDC) prove the presence of two species including <i>Plasmodium vivax</i> and <i>Plasmodium falciparum</i> in this province. <i>P. falciparum</i> cases diagnosis in purpose of true treatment and following up patients is necessary. This study is intending to extract DNA from negative microscopic smears using the newly invented method by research group and afterwards to evaluate negative malaria Giemsa-stained smears using nested polymerase chain reaction.</p> <p>Materials and Methods: Aiming to diagnose malaria, nested PCR amplification was accomplished using DNA extracted from fixed negatively diagnosed microscopic smears and Giemsa-stained. The extracted DNA was used as a pattern to amplify the specific sequence of <i>P. falciparum</i> and <i>P. vivax</i> using the small subunit of ribosomal RNA [18srRNA]. This retrospective study was implemented on 500 malaria negative microscopic smears storing from 6 months to 1 year.</p> <p>Results: By accomplishing PCR amplification on 500 microscopic smears, 54 (10.8%) malaria positive samples were diagnosed which were incorrectly diagnosed to be negative previously. Of all specimens revealed to be positive, 34 (6.8%) cases were <i>P. vivax</i> while 20 (4%) cases were <i>P. falciparum</i>.</p> <p>Conclusion: This study reveals the priority of nested-PCR method to microscopic examination method and the possibility of using old microscopic smears in epidemiologic and retrospective studies clearly.</p> <p>Copyright © 2014 Zahedan University of Medical Sciences. All rights reserved.</p> |

Introduction

Malaria is a major human health-threatening disease, resulting in approximately 300-500 million clinical cases and 1-3 million deaths each year worldwide mainly among young children [1]. One hundred and nine countries were endemic for malaria in 2007 [2]. Iran is situated in the Eastern Mediterranean region, where about 45% of the population lives with the risk of both *P. falciparum* and *P. vivax* malaria. Countries of this region are situated in either Afro-tropical (such as Somalia, Sudan), Oriental (such as Pakistan, south-eastern Iran, part of Afghanistan) or Pale arctic (such as Turkmenistan, Uzbekistan, Tajikistan) eco-epidemiological zones regarding malaria [3]. Sistan and Balouchestan province, South-East of Iran, is the endemic area of malaria and is considered as the oriental eco-epidemiological region of malaria [4]. This province is bordered by Pakistan and Afghanistan. prevalence of malaria has been 938 cases in Iran in 2011 [5]. More than 90% of these cases are reported from Sistan and Balouchestan and Hormozgan provinces [6].

Microscopic examination of Giemsa-stained thick and thin blood smears has been identified as the most common technique to diagnose malaria since last 100 years. Microscopy continues to be the gold standard for identification of Plasmodium spp. in the laboratory setting

[4, 7]. Although easy to apply and cost-effective, this technique assumes that laboratories have certain infrastructure in place with highly skilled professionals and lowest detecting level is 10 to 50 parasites/ μ l, so the sensitivity may fluctuate depending upon the skill of technician [8, 9]. Polymerase chain reaction (PCR) based assays have been used mainly for the assessment of the sensitivity and specificity of microscopy [6]. The PCR method successfully detects parasites in mixed and low level infections, being more sensitive when compared to microscopic examination [10]. PCR-based methods have been shown to be powerful tools for malaria diagnosis [11]. It has been estimated that PCR can detect malaria infections with parasitemia as low as 5 parasites/ μ l (0.0001% parasitemia) [12]. The success of the PCR technique depends on a variety of factors such as: high quality DNA obtained from blood samples, good reagents and adequate conditions of amplification. Whole blood has been shown to be a reliable source of high-quality DNA while Giemsa-stained or unstained thick blood smear and, particularly, blood conserved on filter-papers could be used as a source of DNA in molecular and epidemiological studies [13, 14]. Considering the influence of the quality of biological specimens in malaria molecular diagnosis, the efficacy of PCR in detecting

malaria infection using DNA from blood conserved as Giemsa-stained TBS was evaluated [6]. While routine microscopy might fail to detect very low parasite densities, using modern methods can be useful as a routine method of diagnosis. This study is planned to evaluate microscopy and nested PCR methods on fixed stained films diagnosed to be negative as for detection of malaria parasites.

Materials and Methods

This retrospective study was carried out on a total of 500 historical negative malaria Giemsa-stained smears from malaria department health centers of different districts in Sistan and Baluchestan province. The criteria of sample collection in this study was to be suspected as a malaria patient and diagnosed to be negative for malaria infection in all malaria department health centers. All of the samples following these criteria were included in this study. Many different techniques were tried for DNA preparation from negative fixed and stained slides [6, 15]. All thin and thick blood films were washed with ether and air dried. Subsequently, the whole blood slides were scraped with a sterile scalpel blade into the micro tube containing 20 μ l of 100 mM Na_2HPO_4 and centrifuged at 140000 g for 10 minutes (equivalent to approximately 4 μ l of blood). After three times of repeating this assay then 10 μ l of 5 mM Na_2HPO_4 is added to the pellet and centrifuged for 2 minutes. This step is repeated for two times in the same buffer. The remaining material is resuspended in 10 μ l of sterile water and boiled for 10 minutes. Five micro liter of the supernatant would be used for the PCR assay [6]. Nested PCR amplification was adopted in these experiments, with some modifications for the species-specific gene of Plasmodium species based on the sequence of subunit ribosomal RNA [ssrRNA]. Five micro liter of DNA template was used for the first reaction and 5 μ l aliquot of the first amplification product was then used as a template in a second reaction [16]. In an initial amplification reaction [SPE-NEST 1], the oligonucleotide primer pair: rPLU5 and rPLU6 [5'-TTA AAA TTG TTG CAG TTA AAA CG-3' and 5'-CCT GTT GCC TTA AAC TT-3'] were used. The size of these outer primers is about 1200 bp. The second reaction [SPE-NEST 2] was performed in two separate replicates using two pairs of oligo-nucleotides for detection of two species of Plasmodium parasite. P.fF and P.fR [5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3' and 5'-ACA CAA TGA ACT CAA TCA TGA CTA CCC GTC-3'] for *P. falciparum*, [Size: 205 bp]; P.vF and P.vR [5'-CGC TTC TAG CTT AAT CCA CAT AAC TGA TAC-3' and 5'-ACT TCC AAG CCG AAG CAA AGA AAG TCC TTA-3'] for *P. vivax* [Size: 120 bp].

Results

This study was carried out on 500 historical negative fixed and Giemsa-stained thin and thick slides from different districts of Sistan and Baluchestan province, some stored for at least half a year or more. Nested PCR

amplification for both species of *P. vivax* and *P. falciparum* was performed using ssrRNA. Initial DNA for PCR amplifications was extracted from historical negative fixed and Giemsa-stained slides. Fifty four cases (10.8%) out of 500 historical negative fixed and Giemsa-stained thick and thin slides revealed to be positive 34 (6.8%) cases *P. vivax* and 20 (4%) *P. falciparum* (Fig. 1).

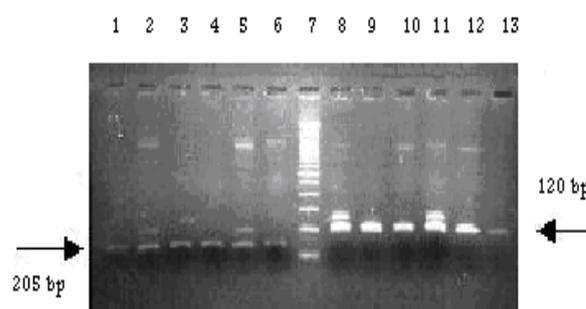


Figure 1. Schematic representation of nested PCR products from clinical specimens by species specific gene of *P. vivax*, *P. falciparum* on 2% agarose gel electrophoresis by 100 bp ladder marker

Discussion

This study showed that of all negative Giemsa-stained slides 54 cases (10.8%) were positive and were diagnosed incorrectly using microscopic examinations. Microscopic examination of Giemsa-stained thick and thin blood smears has been the diagnostic method of choice for species identification in epidemiologic studies and medical diagnosis [17]. The method is simple, does not require highly equipped facilities, and in most cases enables differentiation among the four species causing malaria in humans when performed by an experienced technician. However, this method can sometimes be misleading in identifying parasite species, especially in the case of low level of parasitemia and a mixed parasite infection or modification by drug treatment [4, 18].

In recent years considerable attention has been given to molecular methods, including the PCR techniques [6]. PCR, in particular nested-PCR, is considered to have a promising future in malaria research, especially due to the identification of parasites in areas where four Plasmodium species occur simultaneously [19]. Nevertheless, it has been recognized that the success of the technique depends on the quality of DNA.

It has been observed that intrinsic (as DNA amount or a high content of human DNA or hemoglobin) and extrinsic (use of heparin or inadequate conditions of blood collecting, storage and amplification of samples) are factors affecting the quality of PCR assay [6, 11]. Under some circumstances, PCR amplification of DNA from plasmodia genes may become necessary from infections for which only blood slides are available. We have developed a reliable and controlled method for DNA preparation from malaria parasites on fixed and stained

blood films (thick or thin). A PCR product could be detected using material derived from blood smears. The results of the present study, the use of DNA from thick or thin smears have produced good results by PCR assay in the different studies [20, 21].

All these studies demonstrated that Plasmodium DNA might be successfully isolated from blood smears indicating that this method of DNA preservation could be considered adequate and convenient for epidemiological studies. The results obtained by PCR using isolated DNA from blood samples indicate its great usefulness in field studies. Although false negative results have occurred in our study, the use of isolated DNA from blood allowed the detection of Plasmodium in several samples previously negative by microscopic examination [6]. Routine microscopy fails to detect very low parasite densities. However, malaria prevalence as diagnosed by PCR using DNA extracted from blood films showed a high number of sub clinical parasitaemia (from 500 negative microscopy sample detected by PCR). Those individuals with negative thick and thin blood smears but positive PCR may act as reservoirs of the parasite remains unclear. Although in a malaria endemic area it is most probable that the PCR actually detects infection, a

prospective study performed in the symptom less individuals would be advisable to confirm the infection. This study strongly suggested that preparing and having storage of fixed and stained smears could be useful for epidemiological studies in any endemic area.

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Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

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