

Nested Polymerase Chain Reaction (PCR) on Fixed Stained Slides in Comparison to Whole Blood as a Source of DNA in Southeast of Iran

Ebrahimzadeh Adel^{1*}, Mohammadi Saeed¹ and Polshekan Mir Ali²

¹Department of Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran

²Department of Parasitology and Mycology, Kerman University of Medical Sciences, Kerman, Iran

*Corresponding author: Adel Ebrahimzadeh, Department of Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, Iran, Tel: +989155491303; E-mail: Ebrahimzadeh@zaums.ac.ir

Rec date: Mar 17, 2014, Acc date: Apr 23, 2014, Pub date: Apr 25, 2014

Copyright: © 2014 Adel E, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

The microscopic examination of Thick Blood Smears (TBS) remains the method of choice for the diagnosis of human malaria. Recently, alternative diagnostic methods, such as Nested PCR, have been used for the detection and identification of malaria parasites. The aim of this study was to compare the sensitivity and specificity of Nested PCR accomplished using DNA extracted from whole blood against fixed stained slides. 125 blood samples including 76(60.8%) male and 49(40.2%) female accomplished examinations. The percentage of the parasitaemia on positive samples was calculated from a total count of 200 leukocytes counted in a Giemsa stained thin blood films. The nested PCR assay carried out on DNA Extracted samples by specific primers to amplify 18sr RNA Plasmodium gene. Of all 125 blood samples 50(40%) were positive (41(32.8%) *P. vivax*, 9(7.2%) *P. falciparum*) and 75(60%) were negative for malaria parasites using microscopy examination. Nested-PCR on whole blood specimens detected 66(52.8%) plasmodium species: 47(37.6%) *P. vivax*, 13(10.4%) *P. falciparum*, 6(4.8%) mixed infections *P. vivax* and *P. falciparum*. Nested-PCR on peripheral blood slides detected 49 (39.2%) plasmodium species: 34(27.2%) *P. vivax*, 10(8%) *P. falciparum*, 5(4%) mixed infections *P. vivax* and *P. falciparum*. The study showed that the sensitivity and specificity of nested PCR were 96% and 76%, respectively, when target DNA was extracted from blood and 78% and 86% when DNA was obtained from smears. These studies demonstrated that Plasmodium DNA might be successfully isolated from TBS indicating that this method of DNA preservation could be considered adequate and convenient for epidemiological studies.

Keywords: *Plasmodium vivax*; *Plasmodium falciparum*; Mixed infections; Iran

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 of young children [1]. 109 countries were endemic for malaria in 2007 [2]. Iran is situated in the Eastern Mediterranean region, where about 45% of the population live with the risk of both falciparum and 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].

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 [4,5]. 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 [6,7].

Polymerase Chain Reaction (PCR) based assays have been used mainly for the assessment of the sensitivity and specificity of microscopy. The PCR method successfully detects parasites in mixed

and low level infections, being more sensitive when compared to microscopic examination [8]. PCR-based methods have been shown to be powerful tool for malaria diagnosis [9]. It has been estimated that PCR can detect malaria infections with parasitemia as low as 5 parasites/ μ l (0.0001% parasitemia) [10]. 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 [11,12].

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. This study is planned to evaluate microscopy and nested PCR methods on whole blood and fixed stained films for detection of malaria parasites.

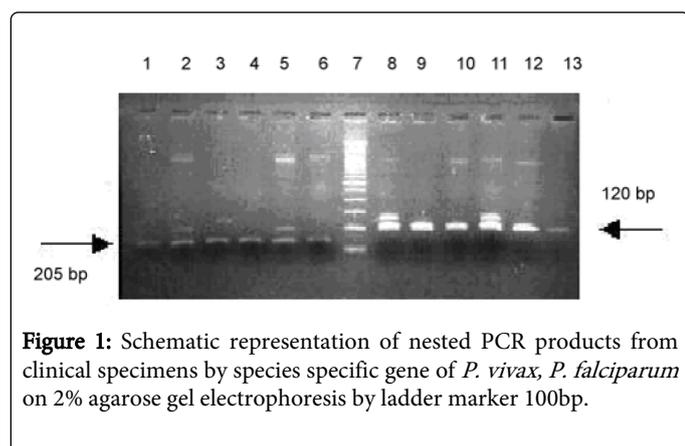
Materials and Methods

This study was carried out on a total of 125 blood samples. Seventy-six individuals (60.8%) were male and 49 individuals (40.2%) were female. Fifty blood samples (40%) diagnosed to be positive, and 75 blood samples (60%) were negative for malaria infection using microscopic methods.

Whole blood samples were mixed with EDTA and sent to Tropical and Infectious Diseases laboratory in Zahedan. Peripheral blood smears (thick and thin) were prepared from the finger prick samples collected aseptically. Thin smears were fixed with methanol at the field condition.

While thick blood smears were air dried. Blood smears were stained with Giemsa staining technique and examined for presence of malarial parasites following standard methodology [13]. Blood smears were interpreted as negative only after examination with an oil immersion lens for at least 100 oil immersion fields by an expert microscopic analyst. The percentage of the parasitaemia on positive samples was calculated from a total count of 200 leukocytes counted in a Giemsa stained thin blood films [14]. Parasites were counted as $\text{Parasite count}/\mu\text{l} = (\text{Total Leukocytes Count} \times \text{Number of infected RBCs}) \div 200$.

Twenty micro litter of positive and negative blood samples treated with 500 μL of phosphate mono sodium 5 mM (NaHPO_4) (PH=8) and DNA extracted by boiling method as described by Foley et al., and according to method described by author, Ebrahimzadeh et al., in 2006 [15,16]. A hundred μl of DNA extracted solution was stored at -20°C until further analysis. Extracted DNA was further amplified for Genus specific amplification. Briefly, it was carried out in a 20 μl reaction mixture consisting 10 \times buffer, 25 mM MgCl_2 , 10 mM dNTPs, 2.5 μM each primers (rPLU6 and rPLU5 to amplify 1050 bp of 18ssu-rRNA), 0.4U of Taq DNA Polymerase, 1.5 μl sample (DNA template). The second round (secondary reaction) of nested PCR for species specific amplification was carried out in two separate tubes each containing a single primer pair (rFAL1, rFAL2 primers to amplify 205bp region of 18ssr RNA *Plasmodium falciparum* and rVIV1, rVIV2 primers amplify 120bp region of 18ssr RNA *Plasmodium vivax*) [17,18] (Figure 1). The PCR amplified DNA product was further analyzed running the products in 2% Agarose gel electrophoresis. The amplified product was visualized using ethidium bromide staining and gel documentation under the UV light [7,16].



Results

From 50 negative blood samples 30(58%) males and 20(42%) female age between 1 to 36 years old. 41(82%) *P. vivax* and 9(18%) *P. falciparum* was reported by microscopy examination. The thin films showed that parasitaemia ranged from 0.008% to 1.8% that indicate parasite is hypo-endemic in this region.

48 (96%) of blood samples was positive for detection of Plasmodium species by PCR assay: 36(72%) *P. vivax*, 8(16%) *P. falciparum*, 4(8%) mixed infections of *P. vivax* and *P. falciparum*,

2(4%) was negative that related to parasitaemia less than 0.009% and PCR sensitivity was 96%.

39(78%) blood positive films have same result for detection of Plasmodium species: 29(58%) *P. vivax*, 7(14%) *P. falciparum*, 3(6%) mixed infections of *P. vivax* and *P. falciparum*, 11(22%) was negative that related to parasitaemia less than 0.03%. and PCR sensitivity was 78%.

From 75 negative blood samples 46(61.3%) males and 29(38.7%) female age between 2 to 50 years old.

PCR on negative blood samples revealed 18(24%) Plasmodium species; 11(14.7%) *P. vivax*, 5(6.7%) *P. falciparum*, 2(2.7%) mixed infections of *P. vivax* and *P. falciparum*, 57(76%) negative and PCR specificity was 76%.

PCR on negative blood samples films revealed 10(13.3%) Plasmodium species; 5(6.7%) *P. vivax*, 3(4%) *P. falciparum*, 2(2.7%) mixed infections of *P. vivax* and *P. falciparum*, 65(86.7%) negative and PCR specificity was 86.6%.

From 125 blood samples 50(40%) positive (41(32.8%) *P. vivax*, 9(7.2%) *P. falciparum*), 75(60%) negative for malaria parasite by microscopy examination. PCR on blood specimens detect 66(52.8%) Plasmodium species; 47(37.6%) *P. vivax*, 13(10.4%) *P. falciparum*, 6(4.8%) mixed infections of *P. vivax* and *P. falciparum*.

PCR on films detected 49 (39.2%) Plasmodium species; 34(27.2%) *P. vivax*, 10(8%) *P. falciparum*, 5(4%) mixed infections of *P. vivax* and *P. falciparum*.

Discussion

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 [19]. 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 [20]. In recent years considerable attention has been given to molecular methods, including the PCR techniques. 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 [21]. 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 [18]. This study has evaluated the sensitivity and specificity of PCR to detect malaria parasites according to the blood conservation devices used in DNA extraction. Considering two alternatives for DNA preparation, the best results (52.8% of prevalence) were obtained for nested PCR analysis of the 18ssuRNA genes when target DNA was isolated from whole blood prepared with EDTA anticoagulant. The study showed that the sensitivity and specificity of nested PCR were 96% and 76%, respectively, when target DNA was extracted from whole blood and 78% and 86% when DNA was obtained from blood smears. This is probably due to the conservation of the biological material used as a source of DNA, which directly affects the quality of

DNA and, consequently, the sensitivity and specificity of the PCR assay. The sensitivity rates below 80% could be explained by the low level of circulating parasites in the blood of the individuals who were part of our study [22,23].

The number of false negative results observed with PCR using DNA from thick blood smears could be an effect of a reduced number of parasites present in the samples, as some could have been lost during the process of scraping the slides. Furthermore, factors involved in preparing slides for microscopic examination may contribute to the stability of DNA template. Classical methods for fixing and storage of cells may be crucial factors in determining the rate of DNA degradation. For example, methanol fixation may affect the dissociation of protein-nucleic acid complexes within the cell. Thus, Giemsa-stain and excessive manipulation of samples could act negatively on DNA integrity. Thus, TBS should be used as a DNA source mainly when a large number of parasites are present on the slides, which will reduce the risk of false negative results enabling the success of the technique [5,16].

In contrast to the results of the present study, the use of DNA from thick or thin smears has produced good results by PCR assay in the different studies [24,25]. All these studies demonstrated that *Plasmodium* DNA might be successfully isolated from TBS indicating that this method of DNA preservation could be considered adequate and convenient for epidemiological studies. A possible explanation for the differences between our results and the above mentioned is the relatively reduced number of parasites present in our samples. On the other hand, 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.

In our study, the use of isolated DNA from whole blood allowed the detection of mixed malaria infections of *Plasmodium vivax* and *Plasmodium falciparum* in several samples previously were negative by microscopic examination. In this case, delayed or missed diagnosis increases the possibility of complicated or severe malaria. Routine microscopy failed to detect very low parasite densities. However, malaria prevalence as diagnosed by PCR using blood DNA showed a high number of sub clinical parasitaemia (from 75 negative microscopy sample detected by PCR). Those individuals with negative thick 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. The goal results of this study exhibited using method of DNA extraction from Stained smears as described by author [16], for following up epidemiological malaria studies in endemic areas.

Acknowledgements

The authors would like to acknowledge Department of Mycology and Parasitology, Faculty of Medicine, Tropical and Infectious Diseases Research Center, Zahedan University of Medical Sciences, Zahedan, Iran for financial supports.

References

1. Hay SI, Okiro EA, Gething PW, Patil AP, Tatem AJ, et al. (2010) Estimating the global clinical burden of *Plasmodium falciparum* malaria in 2007. *PLoS Med* 7: e1000290.
2. (2007) Report of the World Health Organization Technical Consultation on Prevention and Control of Iron Deficiency in Infants and Young Children in Malaria-Endemic Areas, Lyon, France, 12-14 June 2006. *Food Nutr Bull* 28: S489-631.
3. Gething PW, Patil AP, Smith DL, Guerra CA, Elyazar IR, et al. (2011) A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malar J* 10: 378.
4. Berens-Riha N, Sinicina I, Fleischmann E, Löscher T (2009) Comparison of different methods for delayed post-mortem diagnosis of *falciparum* malaria. *Malar J* 8: 244.
5. Ebrahimzadeh A, Fouladi B, Fazaeli A (2007) High rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in South-East of Iran, using nested PCR. *Parasitol Int* 56: 61-64.
6. Makler MT, Palmer CJ, Ager AL (1998) A review of practical techniques for the diagnosis of malaria. *Ann Trop Med Parasitol* 92: 419-433.
7. Ndao M, Bandyayera E, Kokoskin E, Gyorkos TW, MacLean JD, et al. (2004) Comparison of blood smear, antigen detection, and nested-PCR methods for screening refugees from regions where malaria is endemic after a malaria outbreak in Quebec, Canada. *J Clin Microbiol* 42: 2694-700.
8. Singh N, Shukla MM, Shukla MK, Mehra RK, Sharma S, et al. (2010) Field and laboratory comparative evaluation of rapid malaria diagnostic tests versus traditional and molecular techniques in India. *Malar J* 9: 191.
9. Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, et al. (2006) Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum/vivax* endemic area in Thailand. *Malar J* 5: 121.
10. Zalis MG, Ferreira-da-Cruz MF, Balthazar-Guedes HC, Banic DM, Alecrim W, et al. (1996) Malaria diagnosis: standardization of a polymerase chain reaction for the detection of *Plasmodium falciparum* parasites in individuals with low-grade parasitemia. *Parasitol Res* 82: 612-616.
11. Cunha MG, Medina TS, Oliveira SG, Marinho AN, Póvoa MM, et al. (2009) Development of a Polymerase Chain Reaction (PCR) method based on amplification of mitochondrial DNA to detect *Plasmodium falciparum* and *Plasmodium vivax*. *Acta Trop* 111: 35-38.
12. Mayor A, Moro L, Aguilar R, Bardaji A, Cistero P, et al. (2012) How Hidden Can Malaria Be in Pregnant Women? Diagnosis by Microscopy, Placental Histology, Polymerase Chain Reaction and Detection of Histidine-Rich Protein 2 in Plasma. *Clin Infect Dis* 54: 1561-1568.
13. Gao Y, Zhong ZM, Yang PC, Zhang HY, Wei DH, et al. (2012) Improvement of fully automatically microscopic scanning system based on the oil-lens and its application in reading of malaria blood smears. *Chinese journal of schistosomiasis control* 24: 67-71.
14. Dubey ML, Weingken C, Ganguly NK, Mahajan RC (1999) Comparative evaluation of methods of malaria parasite density determination in blood samples from patients & experimental animals. *Indian J Med Res* 109: 20-7.
15. Foley M, Ranford-Cartwright LC, Babiker HA (1992) Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. *Mol Biochem Parasitol* 53: 241-244.
16. Ebrahimzadeh A, Polshakan M, Qureshi M, Sharifi I (2006) Reliable DNA Extraction on Historical Malaria Negative Smears Conducted to Nested PCR in South-East of Iran. *Biotechnology* 5: 353-357.
17. Bharti AR, Letendre SL, Patra KP, Vinetz JM, Smith DM (2009) Malaria diagnosis by a polymerase chain reaction-based assay using a pooling strategy. *Am J Trop Med Hyg* 81: 754-757.
18. Bharti AR, Patra KP, Chuquiyauri R, Kosek M, Gilman RH, et al. (2007) Polymerase chain reaction detection of *Plasmodium vivax* and *Plasmodium falciparum* DNA from stored serum samples: implications for retrospective diagnosis of malaria. *Am J Trop Med Hyg* 77: 444-446.

-
19. Long EG (2009) Requirements for diagnosis of malaria at different levels of the laboratory network in Africa. *Am J Clin Pathol* 131: 858-860.
 20. Perneger TV, Szeless T, Rougemont A (2006) Utility of the detection of Plasmodium parasites for the diagnosis of malaria in endemic areas. *BMC Infect Dis* 6: 81.
 21. Berry A, Benoit-Vical F, Fabre R, Cassaing S, Magnaval JF (2008) PCR-based methods to the diagnosis of imported malaria. *Parasite* 15: 484-488.
 22. Parajuli K, Hanchana S, Inwong M, Pukrittayakamee S, Ghimire P (2009) Comparative evaluation of microscopy and polymerase chain reaction (PCR) for the diagnosis in suspected malaria patients of Nepal. *Nepal Med Coll J* 11: 23-27.
 23. Parkes R, Lo T, Wong Q, Isaac-Renton JL, Byrne SK (2001) Comparison of a nested polymerase chain reaction--restriction fragment length polymorphism method, the PATH antigen detection method, and microscopy for the detection and identification of malaria parasites. *Can J Microbiol* 47: 903-907.
 24. Kimura M, Kaneko O, Inoue A, Ishii A, Tanabe K (1995) Amplification by polymerase chain reaction of Plasmodium falciparum DNA from Giemsa-stained thin blood smears. *Mol Biochem Parasitol* 70: 193-197.
 25. Li J, Wirtz RA, McCutchan TF (1997) Analysis of malaria parasite RNA from decade-old Giemsa-stained blood smears and dried mosquitoes. *Am J Trop Med Hyg* 57: 727-731.