ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (3): 353-357, 2006
ISSN 1682-296X

Reliable DNA Extraction on Historical Malaria Negative Smears Conducted to Nested PCR in South-East of Iran

[1]A. Ebrahimzadeh, [2]M. Polshakan, [1]M.I. Qureshi and [2]I. Sharifi
[1]Zahedan Medical Sciences University,
[2]Department of Parasitology and Mycology, Medical College,
Kerman Medical Sciences University, Zahedan-Iran

Abstract: The reliability of PCR depends on many factors, such as high quality DNA pellets obtained from fixed Giemsa-stained blood samples, good reagents and adequate conditions of amplification. In account of this limitations, a retrospective epidemiological study for malaria diagnosis was conducted in a hypoendemic area, Sistan and Baluchestan province of south-east of Iran. For the first time this research designed to perform a nested PCR protocol using new and modified method of DNA extraction from negative Fixed and Giemsa Stained smears. This study demonstrated 11.55% PCR positive for *Plasmodium vivax* and *Plasmodium falciparum* which were undetectable by microscopy.

Key words: Iran, *Plasmodium falciparum*, *Plasmodium vivax*, nested PCR

INTRODUCTION

Malaria is one of the most important infectious diseases in the third World as well as in Iran. Iran is situated in the south-western of Asia and in the Eastern Mediterranean region (EMR), where about 45% of the population live with the risk of both falciparum and vivax malaria (Sadrizadeh, 2001). Sistan and Baluchestan province is located in the south-eastern of Iran, has been introduced as an endemic area of malaria. Both species of Plasmodium falciparum and vivax has been reported (Zakeri *et al*., 2002). Diagnosis of *P. falciparum* cases is necessary for accurate treatment and following up the patients.

The standard method for detecting *Plasmodium* infection is the microscopic examination of Giemsa-stained thick and thin blood smears. Although effective and inexpensive, such method is laborious and time-consuming and its sensivity drops with the decrease of parasitaemia (Sethabuter, 1992; Barker, 1994; Jelinek *et al*., 1996; Carrasquilla *et al*., 2000).

Recently, Another approach is based on the detection of DNA sequence, like as PCR, have been used for the detection and identification of malaria parasites. The PCR method successfully detects parasites in mixed and low level infections, being more sensitive when compared to microscopic examination proved (Carrasquilla *et al*., 2000), validating its use in malaria epidemiological studies. In this connection, the PCR has become a major diagnostic and research technique. It is also valuable for detection of parasites present at low concentrations in blood or serum samples. For several parasitic species, in particular for plasmodium species, analysis of amplified products encoding polymorphic proteins allowed the discrimination not only of species, but also of subspecies or strains (Babiker *et al*., 1994; Felger *et al*., 1994; Contamin *et al*., 1995; Paul *et al*., 1995). But very often sample material is limited, or material is not available for preparation of DNA. Historical samples, collected many years ago, are available only as fixed and stained microscope slides (Edoh *et al*., 1997). To use such samples for retrospective analysis, we attempted to utilize material isolated from fixed and stained malaria blood films. Microscopy has historically been the mainstay of the diagnosis of malaria. A clinical diagnosis of malaria currently depends on the visualization of parasites by light microscopy of Giemsa-stained thick and thin blood smears. This procedure is cheap and simple, but it is a labour intensive procedure (Payne, 1988).

Many studies have demonstrated the greater sensitivity and specificity of PCR compared to microscopy. The detection of low *P. vivax* and *P. falciparum* parasitaemia by PCR at levels undetectable by microscopy, has been reported (Snounou *et al*., 1993;

Corresponding Author: Dr. A. Ebrahimzadeh, Department of Parasitology and Mycology, Medical College, Zahedan Medical Science University, P.O. Box 98165-494, Zahedan-Iran
Tel/Fax: 0098541 3254799, 0098 09153104393

Roper *et al*., 1996; Singh *et al*., 1996; Zakeri *et al*., 2002). When parasites levels are very low, the information obtained by microscopy is restricted and in some cases biased, by the inability to devote the necessary amount of time to the examination of blood smears. A missed diagnosis of *P. vivax* concurrent with *P. falciparum* is more problematic since these species could cause relapses, thereby compounding morbidity. Because of negative microscopical diagnosis untreated patients may be carriers of the malaria parasites in these particular areas. The number of patients who had traveled to Pakistan, Afghanistan and other parts of Sistan and Baluchestan province were high, with the risk of introducing new isolates (including drug resistant parasites) from neighboring countries.

MATERIALS AND METHODS

This study was carried out in the historical negative fixed Giemsa-stained slides from Malaria Department health centers of the five different districts of Sistan and Baluchestan province (Table 1). Many different techniques were tried for DNA preparation from negative fixed and stained slides (Foley *et al*., 1992; Edoh *et al*., 1997). All thin blood films were washed with ether and air dried. Subsequently, the whole thin blood slides were scraped with a sterile scalpel blade into the micro tube contained 20 μL of 100 mM Na_2HPO_4 and centrifuged at 140,000 g for 10 min (equivalent to approximately 4 μL of blood) (Shute, 1986). After 3 times repeating the assay, then 10 μL of 5 mM Na_2HPO_4 were added to the pellet and centrifuged at 140,000 for 2 min. This part of protocol was repeated 2 times in the same buffer. The remaining material was resuspended in 10 μL of sterile water and boiled for 10 min. Five microliter of the supernatant were used for the PCR assay. Nested PCR amplification as described by Snounou (1993) was adopted in these experiments, with some modifications for the species-specific gene of *Plasmodium* species based on the sequence of their small subunit ribosomal RNA [ssrRNA]. Five microliter of DNA template was used for the first reaction and 5 μL aliquot from the product of the first reaction was then used as a template in a second reaction. 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 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 oligonucleotides for detection of two *Plasmodium* species. 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 carried out with 199 historical negative fixed and Giemsa-stained thin slides from different districts of province, some stored for at least one years (>6 months). Nested PCR amplification for both species of *P. vivax* and *P. falciparum* was performed using ssrRNA. Initial DNA for PCR analyses was extracted from historical negative fixed and Giemsa-stained thin slides. 23 cases (11.55%) out of 199 historical negative fixed and Giemsa-stained thin slides demonstrated PCR positive, 17 cases (8.54%) *P. vivax* and 6 cases (3.01%) *P. falciparum* (Table 1 and Fig. 1a, b).

Table 1: Frequency of PCR results in historical negative fixed Geimsa- stained (> 6 months old) in Sistan and Baluchestan province

Gel results from malaria centers	Chabahar	Iranshahr	Sarbaz	Zahedan	Saravan	Total
PCR negative	41 (87.2%)	37 (84.4%)	42 (87.5%)	28 (90.3)	38 (92.8%)	176 (88.4%)
P.v PCR positive	5 (17.6%)	3 (22.6%)	4 (17.3%)	2 (22.9%)	3 (19.6%)	17 (8.54%)
P.f PCR positive	1 (1.12%)	2 (6.25%)	2 (4.16%)	1 (3.22%)	0 (0%)	6 (3.01%)
Total PCR positive samples	6 (12.76%)	5 (15.62%)	6 (12.5%)	3 (9.7%)	3 (7.31%)	23 (11.55%)

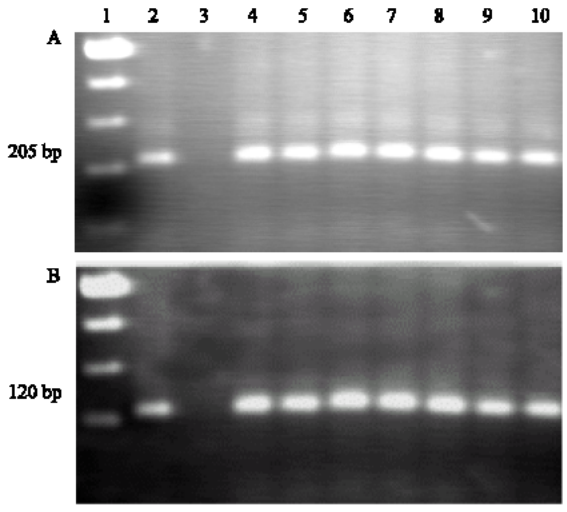

Fig. 1: Schematic representation of agarose gel electrophoresis of nested PCR for A (*P. falciparum*) and B (*P. vivax*), lane 1 ladder marker 100 bp, lane 2 positive control, lane 3 negative control, lane 4-10 PCR positive products

DISCUSSION

In contrast to 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 involving Plasmodium species (Kimura *et al*., 1997; Edoh *et al*., 1997). All these studies demonstrated that DNA might be successfully isolated from TBS (thin or thick blood smear) indicating that this method of DNA preservation could be considered adequate and convenient for epidemiological studies.

It is well know that the success of the PCR assay depends on a variety of factors such as high quality DNA obtained from blood samples, good reagents and adequate conditions of amplification. Under some circumstances, PCR amplification of DNA from Plasmodia gene 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 (Thin smear). A PCR product could be detected using material derived from thin blood films.

This retrospective study has evaluated the sensitivity and specificity of PCR to detect malaria parasites according to the blood conservation devices used for DNA extraction. This is probably due to the conservation of the biological material used as a source of the DNA, which directly affects the quality of DNA and 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 cell 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 (Scopel *et al*., 2004). Whether those individuals with negative 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.

With the spread of parasite resistance to antimalarial drugs (Edrissian *et al*., 1999) in Sistan and Bluchestan province and the increasing difficulty in controlling malaria in these areas, it is important to diagnose malaria accurately and to treat it correctly. Microscopic observation of parasites stained with Giemsa in thin smears is an inexpensive and simple method that is still used in these areas with malaria transmission and where the diagnosis of malaria part of primary health care. Several malaria infections from endemic countries are sub patent, with very low parasitamia and our results also showed this has occurred in this study areas (Zakeri *et al*., 2002).

When parasites levels are very low, the information obtained by microscopy is restricted and in some cases biased, by the inability to devote the necessary amount of time to the examination of blood smears. A missed diagnosis of *P. vivax* concurrent with *P. falciparum* is more problematic since these species could cause relapses, thereby compounding morbidity. Because of negative microscopical diagnosis untreated patients may be carriers of the malaria parasites in these particular areas.

Therefore, an improved DNA purification system had to be used and affinity purification yielded the most sensitive and reliable results. The ability to use Plasmodium DNA from fixed and stained microscope slides for the PCR is very valuable and analysis of genetic markers directly from slides will be useful in certain application. This technique can be used with samples from places where no facility exists for blood collection and strige, such as remote health stations, in basic epidemiological field studies, or by travelers to diagnose their illness retrospectively. Furthermore, in many places microscope slides have been kept for many years and it will be possible to use this valuable historical material to investigate, for example, the spread of drug resistance of *P. falciparum* or other genetic traits, when suitable markers become available. Only a few studies have been conducted on such historical material, all with only limited success and in most cases the techniques were rather cumbersome and unreliable (Kimura *et al*., 1995).

On the other hand, the PCR was able to detect Plasmodial infections which were overlooked by microscopy. It is well established that the PCR, under optimal conditions and with sufficient volume, is better than microscopy at detecting low parasitaemias (Feleger *et al*., 1995).

CONCLUSION

We suggested that DNA isolated from fixed and stained blood films used for malaria diagnosis can be used for PCR analysis. Certainly, this will not be the starting material of choice for large scale studies or accurate assessment of parasite prevalence, when blood samples can be collected conventionally, but it may enable

researchers to study and analyze amplified DNA for several purposes from blood films collected before the development of the PCR.

Final conclusions: Although the blood preserved as thin and thick blood smear provides and alternative and useful tool for malaria molecular diagnosis, its relatively poor performance at low level parasitaemias impairs the correct determination of malaria prevalence in epidemiological studies. However, the results obtained in the present study confirm that the use of fixed Giemsa-stained slides to collect blood is useful for field studies. This research firmly showed that PCR protocol preferable than microscopic method and it might be very useful protocol for epidemiological and retrospective study in an endemic area using historical fixed-stained blood smears. This study strongly suggested that preparing and having storage of no fixed and staining smears is very useful and necessary for epidemiological study in any endemic area.

ACKNOWLEDGEMENTS

Our deep appreciation and thanks to Zahedan and Kerman Medical Sciences Universities for sponsoring this study.

REFERENCES

Babiker, H., L. Ranford-Cartwright and Sultan A. *et al*., 1994. Genetic evidence that RI chloroquine resistance of *Plasmodium falciparum* is caused by recrudescence of resistant parasites. Trans. Royal Soc. Trop. Med. Hyg., 88: 328-331.

Barker, R.H., T. Banchongaksorn, J.M. Courval, W. Suwonkerd, K. Rimwungtragoon and D.F. Wirth, 1994. *Plasmodium falciparum* and *P. vivax*: Factors affecting sensitivity and specificity of PCR-based diagnosis of malaria. Exp. Parasitol., 79: 41-49.

Carrasquilla, G., M. Banguero, P. Sanchez and F. Carvajal *et al*., 2000. Epidemiologic tools for malaria surveillance in an urban setting of low endemicity along the Colombian Pacific Coast. Am. J. Trop. Med. Hyg., 62: 132-137.

Contamin, H., T. Fandeur, S. Bonnefoy and F. Skouri *et al*., 1995. PCR typing of field isolates of *Plasmodium falciparum*. J. Clin. Microbiol., 33: 944-951.

Edoh, D., S. Steiger, B. Genton and H.P. Beck, 1997. PCR amplification of DNA from malaria parasites on fixed and stained thick and thin blood films. Trans. R. Soc. Trop. Med. Hyg., 91: 361-363.

Edrissian, G.H., M. Nateghpoor, A. Afshar, A. Sayedzadeh, G.H. Mohsseni, M.T. Satvat and A.M. Emadi, 1999. Monitoring the response of *Plasmodium falciparum* and *Plasmodium vivax* to antimalarial drugs in the malarious arease in southeast Iran. Arch. Ir. Med., 2: 61-66.

Felger, I., L. Tavul, S. Kabintik, V. Marshall, B. Genton, M. Alpers and H.P. Beck, 1994. *Plasmodium falciparum*: Extensive Polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua new Guinea. Exp. Parasitol., 79: 106-116.

Feleger, I., L. Tavul, A. Narara, B. Genton, M. Alpers and H.P. Beck, 1995. The use of the polymerase chain reaction for more sensitive detection of *Plasmodium falciparum*. Papua New Guinea Med. J., 38: 52-56.

Foley, M., L.C. Ranford-Cartwright and H.A. Babiker, 1992. Rapid and simple method for isolating malaria DNA from fingerprick samples of blood. Mol. Biochem. Parasitol., 53: 241-244.

Jelinek, T., S. Proll, F. Hess, G. Kabagambe *et al*., 1996. Geographic differences in the sensitivity of a Polymerase chain reaction for the detection of *Plasmodium falciparum* infection. Am. J. Trop. Med. Hyg., 55: 647-651.

Kimura, M., H. Miyake, H.S. Kim, M. Tanabe, M. Arai, S. Kawai, A. Yamane and Y. Wataya, 1995. Species-specific PCR detection of malaria parasites by microtiter plate hybridization: Clinical study with malaria patients. J. Clin. Microbiol., 33: 2342-2346.

Kimura, M., O. Kanoek, M. Zhou, F. Kawamoto, Y. Wataya, S. Otani, Y. Yamaguchi and K. Tanabe, 1997. Identification of the four species of human malaria parasites by nested PCR that targets variant sequences in the small subunit rRNA gene. Parasitol. Intl., 46: 91-95.

Roper, C., I.M. Elhassan, L. Hviid, H. Giha, W. Richardson, H. Badiker, G.M. Satti, T.G. Theander and D.E. Arnote, 1996. Detection of very low level *Plasmodium falciparum* infections using the nested polymerase chain reaction and a reassessment of the epidemiology of unstable malaria in sudan. Am. J. Trop. Med. Hyg., 54: 325-331.

Sadrizadeh, B., 2001. Malaria in the world, in the eastern Mediterranean region and in Iran: Review article. WHO/EMRO Report, 1-13.

Scopel, K.K.G., C.J.F. Fontes, A.C. Nunes, M.D.F. Horta and E.M. Braga, 2004. Low sensitivity of nested PCR using Plasmodium DNA extracted from stained thick blood smears: An epidemiological retrospective study among subjects with low parasitaemia in an endemic area of the Brazilian Amazon region. Malaria J., 31: 3-8.

Sethabuter, O., A. Brown, S. Panyim, K.C. Kain, H.K. Webster and P. Echeverria, 1992. Detection of *Plasmodium falciparum* by polymerase chain reaction in a field study. J. Infect. Dis., 166: 145-146.

Shute, G., 1986. The Microscopic Diagnosis of Malaria. In: Malaria, Wernsdorfer, W. and I. McCregor (Eds.). Edinburgh: Churchill Livingstone, pp: 781-814.

Singh, B., J. Cox-Singh, A.O. Miller, M.S. Abdullah, G. Snounou and H.A. Rahman, 1996. Detection of malaria in malaysia by nested polymerase chain reaction amplification of dried blood spots on filter papers. Trans. R. Soc. Trop. Med. Hyg., 90: 519-521.

Snounou, G., S. Viriyakosol, X.P. Zhu, W. Jarra, L. Pinheiro, V.E. Do Rosario, S. Thaithhong and K.N. Brown, 1993. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction in a field study. J. Infect. Dis., 166: 145-148.

Paul, R.E., M.J. Packer, M. Walmsley, M. Lagog, C.L.C. Ranford, R. Paru and K.P. Day, 1995. Mating patterns in malaria parasite populations of Papua New Guinea. Science, 269: 1709-1711.

Payne, D., 1998. Use and limitation of light microscopy for diagnosing malaria at the primary health care level. Bull World Health Organ, 66: 612-629.

Zakeri, S., N.S. Talebi, A. Zare and D.N. Dinparast, 2002. Detection of malaria parasites by nested PCR in South-eastern, Iran: Evidence of highly mixed infections in Chahbahar district. Malaria J., 1: 1-6.