

Real Time Polymerase Chain Reaction for the Detection of Malarial Parasite

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ABSTRACT

Objective: To evaluate the sensitivity and specificity of real time polymerase chain reaction (PCR) for the detection of Malarial parasite.

Study Design: Descriptive cross-sectional study.

Place and Duration of Study: The Armed Forces Institute of Pathology (AFIP), Rawalpindi, from April to June 2010.

Methodology: A total of 60 Leishman stained blood films with clinical suspicion of malaria were studied by light microscopy for detection of malaria parasite (MP). The samples were also subjected to real time PCR for the small subunit (SSU) rRNA gene of MP found in all the four subspecies of *Plasmodium*. Real time PCR was done by the Taqman probe method. One sample positive for MP was serially diluted with ABO compatible blood, and light microscopy and real time PCR were performed on all dilutions. Results of light microscopy and real time PCR were compared. Sensitivity and specificity were calculated using PCR as the gold standard.

Results: PCR detected MP in 33 samples with sensitivity and specificity of 100% whereas light microscopy could detect MP in 30 samples. Sensitivity and specificity of light microscopy was 90.9% and 100% respectively. In the serially diluted blood sample, MP was visible at 1/16 dilution whereas the PCR showed positive results even at 1/512 dilution.

Conclusion: Real time PCR is more sensitive than light microscopy for the detection of malarial parasite.

Key words: Malaria. Real time polymerase chain reaction. Light microscopy.

INTRODUCTION

Malaria is a protozoan infection caused by four species of *Plasmodium* (*P.*), namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. It is characterized by intermittent high grade fever, splenomegaly, thrombocytopenia and anaemia. Serious life threatening complications can occur with multiple organ involvement in *P. falciparum* infection.¹ Malaria is responsible for 300-500 million clinical cases and nearly one million deaths per year worldwide, the majority of which are children under 5 years of age.² Pakistan was included in world's 30 high burden malaria countries by World Health Organization (WHO) in 2008.³ Around 60,000 confirmed malaria cases were reported in Pakistan in 2008, 30% of which were due to *P. falciparum*.⁴

The laboratory methods used to diagnose malaria include peripheral blood film microscopy, rapid antigen detection tests, molecular techniques like PCR, quantitative buffy coat examination and serological tests.⁵ Light microscopy is considered to be the gold

standard for detection of the malarial parasite (MP) because it implies direct visualization of MP but it requires expertise, is laborious and time-consuming.⁶ A single negative blood film does not exclude malaria,¹ and thick blood film underestimates parasite density.⁷ This reduces the sensitivity of light microscopy particularly when in inexperienced hands.

Studies have shown that PCR is more sensitive than light microscopy in detection of MP, especially in low parasitaemia.⁸⁻¹¹ PCR is also useful in detecting asymptomatic malaria, and evaluation of anti-malarial therapy and drug resistance.^{9,10} PCR is costly but the expenses can be substantially reduced by developing in-house PCR reagents.

The aim of this study was to evaluate the sensitivity and specificity of an in-house real time PCR for malaria as compared to the morphology by light microscopy.

METHODOLOGY

This study was conducted at the Department of Haematology, Armed Forces Institute of Pathology (AFIP), Rawalpindi, from April to June 2010. A total of 60 samples submitted to AFIP with clinical suspicion of malaria were studied.

Three ml venous blood was collected in EDTA. The blood samples were used for preparing blood smears and extraction of DNA for PCR. Blood smears were stained with Leishman stain and microscopy was performed for detection of MP. DNA was extracted by

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using Puregene genomic DNA purification kit, Gentra systems, USA. Small sub-unit (SSU) rRNA gene found in all four subspecies of *Plasmodium* was used as the target for PCR amplification. The real time PCR was done by the Taqman probe method as described by Lee *et al.*¹² Known positive samples previously diagnosed as malaria and negative samples from uninfected individuals were used as controls. The sequence of the forward and reverse primers, and the Taqman probe were as follows:

Forward: 5'-ACATGGCTATGACGGGTAACG-3'
 Reverse: 5'-TGCCTTCCTTAGATGTGGTAGCTA-3'
 Probe: 6FAM5'-TCAGGCTCCCTCTCCGGAATCGA
 -3'-TAMRA

Real time PCR amplification was done on ABI (USA) machine 7500. PCR was carried out in a 25 µl reaction mixture containing 1 µl primer mix, 0.1 µl Taq polymerase, 22 µl PCR mix and 2 µl genomic DNA. The thermal cycling consisted of initial denaturation at 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, primer annealing and DNA extension reaction at 60°C for 1 minute.

One of the samples positive for MP was serially diluted with ABO compatible blood. Light microscopy and PCR for MP was carried out on each dilution. Results of light microscopy and real time PCR were compared. Sensitivity and specificity were calculated using PCR as the gold standard.

RESULTS

Out of the 60 samples, light microscopy could detect MP in 30 samples, out of which 27 were infected with *P. vivax*, one with *P. falciparum* while two had mixed *P. vivax* and *P. falciparum* infection. PCR detected MP in 33 samples including 3 samples that were negative for MP on light microscopy; however, identification of *Plasmodium* species was not possible because PCR was not species specific. Sensitivity of light microscopy and PCR was 90.9% and 100% respectively. Specificity of both methods was 100%.

Malarial parasite was detected in serially diluted sample till 1/16 dilution on light microscopy and 1/512 dilution on real time PCR, showing that PCR could detect five folds low parasitaemia as compared to microscopy.

DISCUSSION

Malaria is one of the most common parasitic infections of human and poses a major health threat. Signs and symptoms of Malaria have poor specificity, thus clinical features are of limited use in its diagnosis.¹³ Overuse of antimalarials is not only leading to drug resistance but also wastage of resources. Therefore, accurate diagnosis of Malaria is essential for treatment, and prevention of life threatening complications. Although

light microscopy is still the method of choice for the diagnosis of Malaria because it is simple and cost effective. However, it lacks sensitivity and may give false negative results.

In this study, the sensitivity of real time PCR was higher than microscopy and it was able to detect considerably low level of parasitaemia that was not detected by light microscopy. Similar results have been reported previously in various studies,^{4,6} but published data on this topic is not available from Pakistan. PCR is useful in detecting Malaria infection missed on light microscopy. It can also diagnose suspicious cases where clinical features suggest Malaria but light microscopy fails to demonstrate the parasite.

In countries where Malaria is not endemic, PCR can be useful as primary diagnostic tool without need of multiple sampling for light microscopy, leading to correct diagnosis and avoiding overtreatment with anti-malarials.^{8,14} On the other hand, in countries where Malaria is endemic, limited resources coupled with inadequate laboratory infrastructure and expertise makes it difficult to use PCR in routine diagnosis of Malaria.^{11,15} Therefore, real time PCR may be used as a confirmatory test in reference laboratories in Pakistan but microscopy will remain the mainstay of routine malaria diagnosis.

In Pakistan, blood and blood products are either not screened in routine for MP or light microscopy is used, with a considerable risk of transfusion transmitted malaria, if missed on microscopy.^{16,17} PCR has been reported more sensitive to microscopy in detecting MP in blood donors.¹⁸ Therefore, real time PCR for malarial parasite can be used as screening method in blood banks, thus minimizing risk of transfusion transmitted malaria.

In this study, PCR was not species specific, thereby limiting its utility in species identification and diagnosis of mixed infection. This highlights need for further research in this direction to get maximum help from a powerful diagnostic tool.

CONCLUSION

Real time PCR for malarial parasite is more sensitive than light microscopy in detection of Malaria. It is useful in suspected Malaria cases and those having low parasitaemia that are likely to be missed on light microscopy. Its use in Pakistan may be limited as a confirmatory tool in reference laboratories and donor screening in blood banks due to cost and technical inadequacy.

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