# Species-specific diagnosis of human malaria by the polymerase chain reaction

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## Abstract

The polymerase chain reaction (PCR) with species-specific primers derived from a number of *Plasmodium* genes has been shown to be highly specific and sensitive for malaria diagnosis. In this study, nucleotide sequences derived from the mitochondrial cytochrome c oxidase III (COIII) genes of *P. falciparum* and *P. vivax* were used in the PCR to develop species-specific diagnostic assays. *P. falciparum* primets F1 and F2 only amplified *P. falciparum* template DNA while *P. vivax* primers V1 and V2 amplified both DNA templates of *P. vivax* and *P. cynomolgi*. The sensitivity of the PCR with F1 and F2 primers was 0.5 parasite per PCR reaction. PCR performed on DNA samples from 58 malaria patients and 30 uninfected individuals using both primer sets showed that both assays gave 100% sensitivity and 100% specificity. Three samples from patients with mixed infections of *P. falciparum* and *P. vivax* were also positive in both the PCR assays with the two primer sets. The ease of the DNA preparation and PCR procedures used in this study makes this test potentially useful for malaria diagnosis in the field, particularly where both *P. falciparum* and *P. vivax* occur together.

Key Words: PCR; malaria diagnosis; mitochondrial cytochrome *c* oxidase III gene

## Introduction

Studies have demonstrated that the polymerase chain reaction (PCR) with species-specific nucleotides derived from a number of *Plasmodium* genes as primers is highly specific and sensitive for malaria diagnosis. These include studies on the ssrRNA of *Plasmodium falciparum* (Khoo *et al.*, 1996) and other human malaria parasites such *P. vivax*, *P. malariae* and *P. ovale* (Snounou *et al.*, 1993), the circumsporozoite gene of *P. falciparum* and *P. vivax* (Brown *et al.*, 1992; Sethabutr *et al.*, 1992), *P. falciparum* DHFR-TS gene (Wataya *et al.*, 1993) as well as repetitive genomic DNA sequences of *P. falciparum* (Barker *et al.*, 1992; Tirasophon *et al.*, 1991). However, higher sensitivity of PCR assays may be obtained by using nucleotide sequences derived from mitochondrial DNA as the copy number of the mitochondrial DNA is high as shown, for example in *P. yoelli* (Vaidya and Arasu, 1987). In this study, nucleotide sequences derived from the mitochondrial cytochrome c oxidase III (COIII) genes of two human malaria parasites, *P. falciparum* and *P. vivax*, were used as primers in the PCR for detection of the parasites in the infected blood. Previous studies have reported that the nucleotide sequence of the COIII gene of *P. vivax* differs to some extent from that of *P. falciparum* (DDBJ Accession No. 82020, 1996; Lim *et al.*, 1995).

## Materials and Methods

### *Blood samples*

Whole blood was collected from 29 patients infected with *P. falciparum*, 26 patients infected with *P. vivax*, 1 patient infected with *P. malariae* and 3 patients with mixed infections of *P. falciparum* and *P. vivax* from Kuala Lumpur Hospital and Gombak Hospital, Selangor Darul Ehsan, Malaysia. Malaria diagnosis of the patients was made by microscopic examination of 200 fields at 1,000x magnification, of the Giemsa-stained thick blood films for the parasites. The malaria species were identified on the Giemsa stained thin blood films. All slides were examined by two independent microscopists.

Blood samples for the other malaria parasites were collected from experimentally infected animals. These included *P. cynomolgi* and *P. inui* from laboratory-infected *Macaca fascicularis*, *P. yoelli* and *P. berghei* from infected BALB/c mice and *P. gallinaecium* from infected chickens. Blood from 30 uninfected human donors collected by venipuncture in EDTA tubes served as negative controls while two isolates of *P. falciparum* cultured *in vitro* (K1 strain and a local isolate ST 234) served as positive controls in the PCR assay for *P. falciparum*.

### *Extraction of DNA from blood samples*

Whole blood, 60 µl, collected in EDTA tubes, was mixed with 900 µl of phosphate buffered saline (PBS), pH 7.2, and 108 µl of 1.5% saponin in PBS in an eppendorf tube. The mixture was incubated at 37°C for 20 minures, then centrifuged at 10,000 rpm for 10 minutes at 4°C. PBS, 1.2 ml, was added to the pellet, the mixture vortexed briefly and centrifuged at 10,000 rpm for 10 minutes at 4°C. The pellet was washed twice as described above, then dried by using a vacuum concentrator (Savant) and stored at -20°C until use in the PCR assay. Before use, the pellet was resuspended in 20 µl of 10 mM Tris-0.1 mM EDTA, pH 7.4, and heated at 65°C for 5 minutes in a dry bath.

*Determination of sensitivity of the PCR*

The sensitivity of the PCR with *P. falciparum* primers was determined by using the limiting dilution method by Snounou *et al.* (1993). DNA was extracted from 500 µl of *P. falciparum* cultured *in vitro*, with a known parasite count, then ten-fold serial dilutions were made in normal blood of the same specimen. One microlitre of of each dilution was used in the PCR as described above.

*Polymerase chain reaction (PCR)*

PCR was carried out by using the two primer sets derived from the COIII genes of *P. falciparum* and *P. vivax* (Lim *et al.*, 1995). The four primers used in the PCR were: *P. falciparum* specific primers F1 (5'-CTAGAGATTTCAAAACTCATTCC-3') and F2 (5'-GTTTCATATCCTG-CAATTAACATC-3'), *P. vivax* specific primers V1 (5'-GTATCTTATCCTTCATTAA-CATC-3') and V2 (5'-CTAGAAATTTCTAAACT-CATTCC-3'). Both primer sets amplified a 374 bp PCR product. PCR, in 50 µl, was performed with 1 µl of DNA solution derived from each DNA pellet, 50 pmol of each primer, 5 µM of each dNTP, 2.5 units of *Tth* DNA polymerase (Toyobo, Japan), 1 mM Tris-HCl (pH 8.8), 30 mM KCl, 0.1 mM DTT, 0.01 mM EDTA, 50 µg/ml BSA and 5% (v/v) glycerol. PCR with primers F1 and F2 was carried out with denaturation at 94°C for 15 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 10 seconds for 30 cycles. For the PCR with primers V1 and V2, the same conditions were used except that annealing was performed at 62°C for 30 seconds. The PCR products were resolved with 1% agarose gel electrophoresis, stained with ethidium bromide and photographed. The presence of a PCR product of 374 bp was considered as a positive result.

## Results

Results of the PCR assays carried out on all blood samples with the two primer sets on the various species of malaria DNA showed that only *P. falciparum* template DNA was amplified with the *P.falciparum* primers F1 and F2 (Fig. 1), and primers from *P.vivax* COIII amplified both DNA remplates of *P. vivax* and *P. cynomolgi* (Fig.2). Table 1 summarizes the results of the PCR assays performed with DNA samples from 58 malaria patients and 30 uninfected individuals. All of the 30 samples from uninfected individuals were negative in both PCR assays while the 29 *P. falciparum* - infected patient samples were positive with primers F1 and F2 but negative in the PCR with primers V1 and V2. All of the 26 *P. vivax* - infected patient samples were positive in the PCR with primers V1 and V2 but were negative with primers F1 and F2. Therefore when compared with microscopy, both the PCR assays gave 100% sensitivity and 100% specificity. The three samples from patients with mixed infections ( *P. falciparum* and *P. vivax* ) were positive in both the PCR assays with the two primer sets.

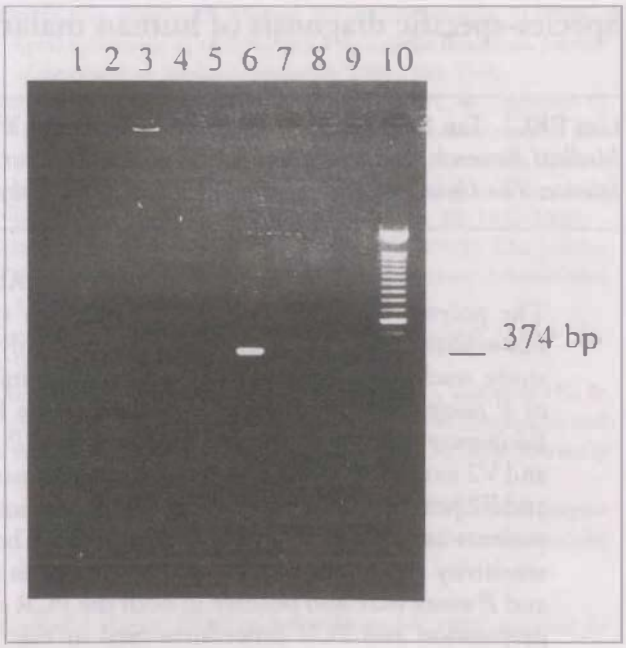


Fig. 1. The results of the PCR with primers F1 and F2, demonstrating the 374-bp amplified product of *P. falciparum* DNA after gel electrophoresis and ethidium bromide staining. Lane 1: *P. berghei* DNA; Lane 2: *P. cynomolgi* DNA; Lane 3: *P. gallinaecium* DNA; Lane 4: *P. inui* DNA; Lane 5: *P. yoelli* DNA; Lane 6: *P. falciparum* DNA; Lane 7: Human DNA; Lane 8: *P. malariae* DNA; Lane 9: *P. vivax* DNA; Lane 10: DNA markers (100 bp ladder).

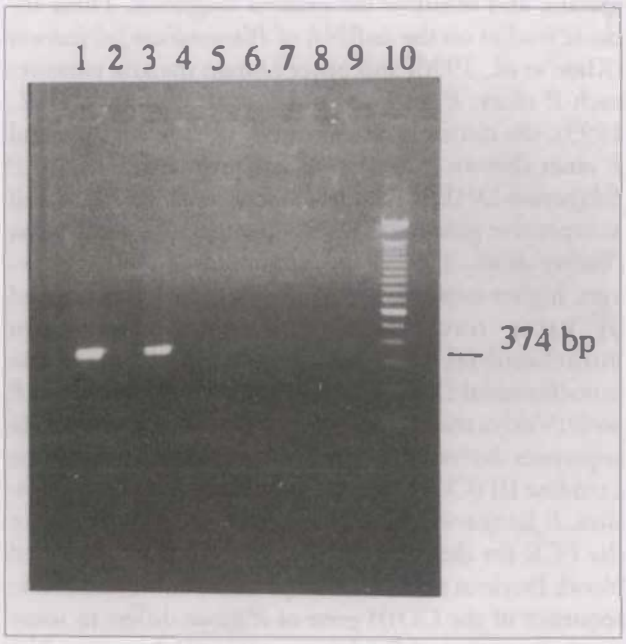


Fig. 2. The results of the PCR with primers V1 and V2, demonstrating the 374-bp amplified products of *P. vivax* and *P. cynomolgi* DNA after gel electrophoresis and ethidium bromide staining. Lane 1: *P. vivax* DNA; Lane 2: *P. falciparum* DNA; Lane 3: *P. cynomolgi* DNA; Lane 4: *P. inui* DNA; Lane 5: *P. yoelli* DNA; Lane 6: *P.gallinaecium* DNA; Lane 7: Human DNA; Lane 8: *P. malariae* DNA; Lane 9: *P. vivax* DNA; Lane 10: DNA markers (100 bp ladder).

Table 1. Comparison of diagnosis of *Plasmodium falciparum* and *Plasmodium vivax* infection by PCR and microscopy.

| | | Diagnostic Method | | | |
|---|---|---|---|---|---|
| | | Microscopy | | PCR | |
| Sample | No. | Parasitaemia/µl Asexual | Sexual | Primers F1-F2 | Primers V1-V2 |
| *P. falciparum* | 29 | 40-362,080 | 0-320 | 29* | 0* |
| *P. vivax* | 26 | 40-40,840 | 0-3,600 | 0* | 26* |
| *P. falciparum* and *P. vivax* | 3 | 3,200-10,560 | 0-1,120 | 3* | 3* |
| Negative | 30 | 0 | 0 | 0* | 0* |

* Each numeral represents the number of DNA samples which were positive in PCR

From the results of the sensitivity test, the lowest parasite detection level was 0.5 parasite per PCR reaction.

## Discussion

In our study, the sample preparation was relatively simple to perform and further studies are being carried out to experiment the use of parasite DNA extracted from blood collected on filter paper to facilitate the use of these assays for field application.

The PCR assays for both *P. falciparum* and *P.vivax* diagnosis were specific for the human infections as DNA from the other human *Plasmodium* spacies was not amplified. However, the PCR with *P. vivax* primers amplified DNA from a simian malaria parasite, *P. cynomologi*. This should not pose a serious problem in most instances as *P. cynomolgi* has been reported to occur only as incidental infections in humans (Schmidt *et al.*, 1961).

In our study, we used *Tth* DNA polymerase instead of *Taq* DNA polymerase and this renders the assay cheaper for field application. The sensitivity of our assay with *P. falciparum* primers was comparable to that reported by several workers (Barker *et al.*, 1993; Sethabutr *et al.*, 1992; Wataya *et al.*, 1993) although higher levels of sensitivity have been reported in other studies (Khoo *et al.*, 1996; Tirasophon *et al.*, 1992). However, in our study, for the three cases of mixed infections which were predominantly *P. falciparum*, the PCR assay with *P. vivax* primers was sensitive enough to detect the low numbers of *P. vivax*. In fact, for two of these three samples, one of the microscopists reported only *P. falciparum*.

This study examined a small number of samples and the specificity of both the assays was high. However, this needs further confirmation with a larger sample size. Based on the ease of the DNA preparation and the simplicity of the PCR procedures, we conclude that our PCR assays with species-specific nucleotide sequences from the mitochondrial COIII genes of *P. falciparum* and *P. vivax* are potentially useful for malaria diagnosis in the field, particularly where both *P. falciparum* and *P. vivax* occur together.

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