Short Report

Diagnosis of *Plasmodium malariae* infection by the polymerase chain reaction

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Hybridization with species-specific probes and the polymerase chain reaction have been used to identify species of malaria parasites. Hybridization is specific, but the sensitivity of the probes may not surpass that of microscopical examination of thick blood films (BARKER *et al.*, 1989). Hybridization techniques are also time-consuming and, in the published studies, use highly radioactive ^{32}P. The polymerase chain reaction is not radioactive, more rapid to perform and more sensitive than hybridization, and is thus more suitable for application in malaria-endemic countries with limited laboratory resources (BARKER, 1994).

Previous investigations of the polymerase chain reaction-based diagnosis of human malaria (without hybridization on a nylon membrane) have been focused on the detection of *Plasmodium falciparum* and *P. vivax* (see LAL *et al.*, 1989; ARAI *et al.*, 1994; TIRASOPHON *et al.*, 1994). Only a few studies have described a polymerase chain reaction method to identify *P. malariae*, using the small subunit ribosomal ribonucleic acid (rRNA) gene as the molecular target (SNOUNOU *et al.*, 1993a, 1993b). In the present study, our aim was to develop an alternative, specific polymerase chain reaction method to detect *P. malariae*, using a species-specific region of another molecular target, the circumsporozoite protein (CSP) gene of *P. malariae*.

Venous blood was drawn from symptomatic indigenous patients attending the Nlongkak dispensary in Yaoundé, Cameroon, if microscopical examination of a thick blood film had revealed the presence of *P. malariae*. The parasitaemias ranged from 0·08 to 0·8%. Blood samples were washed twice in RPMI-1640 medium and part of the buffy coat was removed. Blood samples (1 mL) were lysed with saponin and treated with 1% Sarkosyl™, ribonuclease A, and proteinase K. Genomic deoxyribonucleic acid (DNA) was extracted with phenol and precipitated in ethanol. The approximate yield of total DNA obtained from 1 mL of red cell pellet was 5–15 µg dissolved in 25–50 µL of TE buffer (10 mM Tris/1 mM ethylenediaminetetraacetic acid).

The initial polymerase chain reaction was performed using a PTC-100™ thermal cycler (MJ Research Inc., Watertown, Massachusetts, USA) under the following conditions: approximately 1·5 µg of total DNA (*P. malariae* DNA contaminated with human leucocyte DNA in 2–8 µL of TE buffer), 15 pmol of each of the primer pair PMCSP-1 (5′-AAGAAGTTATCTGTCTTAGCAATATCC-3′; sense) and PMCSP-2 (5′-TTAGTGAAAGAGTATTAAGACTAAAAC-3′; antisense), buffer (50 mM KCl, 10 mM Tris, pH 8·4), 1·5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (dNTP), and 1 unit of *Taq* DNA polymerase in 50 µL reaction mixture; 94°C×5 min for the first cycle and 1 min in subsequent cycles, 50°C×5 min for the first cycle and 1 min in subsequent cycles, and 72°C×5 min for the first cycle and 2 min in subsequent cycles, for a total of 30 cycles. The primers PMCSP-1 and PMCSP-2 were designed on the basis of full-length sequences of the *P. malariae* CSP gene (LAL *et al.*, 1988; QARI *et al.*, 1994). The *P. malariae* CSP sequence was aligned and compared with that of other human and rodent malaria parasites to select species-specific conserved nucleotide sequences at the 5′ and 3′ ends of the gene. The expected size of the fragment amplified by the primer pairs PMCSP-1/PMCSP-2 was 1·3 kilobasès (kb).

To improve the sensitivity of the assay, the quantity of total DNA (parasite DNA and human DNA) obtained from the isolate with the lowest parasitaemia in our study (0·08%) was calculated from optical density readings with an ultraviolet spectrophotometer. A known quantity of total DNA was diluted in distilled water to obtain a range of DNA samples containing between 0·015 and 400 ng. Two internal primers, PMCSP-4 (sense; 5′-TTGAAACAACCCCCCGGA-3′) and PMCSP-5 (antisense; 5′-ATCAACCTTTCTTCTAGC-3′), that hybridized within the conserved regions flanking the central, variable tandem repeat region were designed for the secondary polymerase chain reaction. The nested polymerase chain reaction was performed under the same conditions as the primary polymerase chain reaction, using 2 µL of amplification product from the primary reaction and the primer pair PMCSP-4 and PMCSP-5. Polymerase chain reaction techniques using primers specific for the small subunit rRNA gene, as described by SNOUNOU *et al.* (1993a), were performed to exclude mixed infections with other malaria species. Human DNA was used as a negative control. The amplified product was resolved by 1% agarose gel electrophoresis and stained with ethidium bromide for visual detection by ultraviolet transillumination.

The direct polymerase chain reaction technique described was specific and reproducible with 13 isolates of *P. malariae*. A typical result for 7 clinical isolates of *P. malariae* is shown in Fig. 1. There was no false negative result. The polymerase chain reaction conditions described above are stringent and seem to be optimal for the direct detection of *P. malariae* in a single polymerase chain reaction assay by ethidium bromide staining, without the use of probes or a secondary, nested polymerase chain reaction. Partial DNA sequencing of the fragments amplified by the primers PMCSP-1 and PMCSP-2 and comparison with the published sequenc-

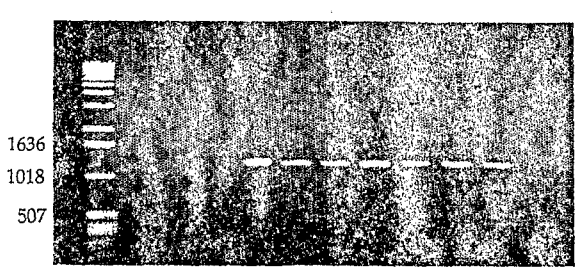

Fig. 1. Circumsporozoite protein gene fragments (1·3 kb) amplified by polymerase chain reaction using genomic DNA of *Plasmodium malariae* after electrophoresis in 1% agarose gel and ethidium bromide staining. Lane L, 1 kb DNA 'ladder' showing M_r values; lane 1, *P falciparum* (FCR3 strain); lane 2, *P. vivax*; lane 3, *P. ovale*; lanes 4–10, Cameroonian isolates of *P. malariae*.

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es confirmed the highly conserved sequences at the 5′ and 3′ ends and characteristic tandem repeats in the central region of the gene (unpublished data). The primer pairs used in this study did not amplify any DNA fragment from *P. falciparum, P. vivax,* or *P. ovale.* The negative results with *P. falciparum* and *P. vivax* were expected because of the CSP gene sequence differences. Although the *P. ovale* CSP gene sequence has not been published, our negative results suggest sequence differences at the 5′ and 3′ ends where the primers hybridize. These observations suggest that the intra-species conserved regions and inter-species variation at the 5′ and 3′ ends of the CSP gene may be useful for species diagnosis by polymerase chain reaction.

A single poylmerase chain reaction assay using the primers PMCSP-1 and PMCSP-2 was sufficient to detect 0·08–0·8% parasitaemia (approximately 1·5 μg of total parasite and human DNA) with all 13 isolates. To determine the sensitivity of the assay, a primary polymerase chain reaction using PMCSP-1 and PMCSP-2 was performed on a serial dilution of the total DNA ranging from 15 pg to 400 ng. The primary assay did not yield any visible band from these diluted genomic DNA samples (results not shown). The sensitivity of the assay to detect *P. malariae* was considerably improved by the nested polymerase chain reaction using 1/25 of the primary amplification product. The secondary nested assay yielded DNA fragments with the expected band size (approximately 860 base pairs) in all diluted samples (Fig. 2).

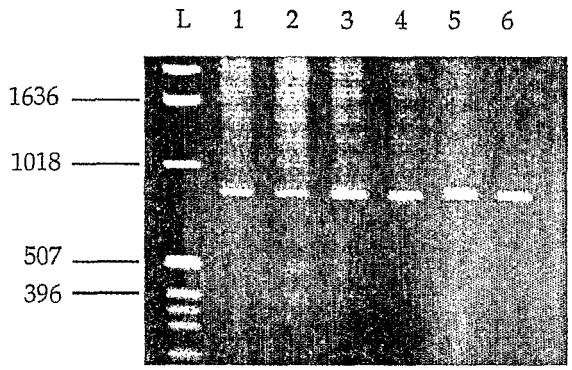

Fig. 2. Nested polymerase chain reaction using 2 μL of the primary amplification product. The primary reaction was carried out using the following quantities of total DNA (*P. malariae* DNA contaminated with human leucocyte DNA): 400 ng (lane 1), 100 ng (lane 2), 10 ng (lane 3), 1·5 ng (lane 4), 150 pg (lane 5) and 15 pg (lane 6). Lane L, 1 kb DNA 'ladder' showing M_r values.

The specific diagnosis of malaria parasites is currently done by microscopical examination of blood films. Most malaria infections are easily recognized by trained microscopists. It may therefore be objected that polymerase chain reaction-based assays for *P. malariae* may not be indicated in most patients for economic reasons, since this parasite is not associated with severe morbidity or fatal disease (except in rare cases of quartan nephrotic syndrome) and rapid, accurate, and inexpensive Giemsa-staining methods already exist. However, there are several applications of polymerase chain reaction-based diagnosis of malaria species. Some cases may pose diagnostic problems, as with patients presenting the low parasitaemia usually associated with *P. malariae* infections. In these cases, the polymerase chain reaction may be a helpful complementary test to establish the diagnosis before appropriate treatment. Even if a rapid DNA extraction method is used, at present diagnosis based on the polymerase chain reaction takes several hours to perform. The time lapse is generally not critical for the treatment of pure *P. malariae* infections. Another possible application of the polymerase chain reaction-based diagnostic technique described in this report is an epidemiological survey of *P. malariae* that involves screening a large number of blood samples.

In conclusion, we have developed an alternative method of polymerase chain reaction-based diagnosis of *P. malariae* infection using the CSP gene as the DNA target. A single 30-cycle amplification was sufficient to detect 0·08–0·8% parasitaemia. The sensitivity of the assay, based on visual DNA detection by ethidium bromide staining, was considerably improved by the use of a secondary nested polymerase chain reaction. The method is specific and may be useful as an adjunct to clinical diagnosis as well as a tool for large-scale screening for *P. malariae.*

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