Malaria: even more chronic in nature than previously thought; evidence for subpatent parasitaemia detectable by the polymerase chain reaction

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Abstract

In high endemicity areas, malaria is a chronic disease: examination of blood films reveals that up to half of the population, particularly children, harbour parasites at any one given time. The parasitological status of the remainder was addressed using the polymerase chain reaction, a technique 100 to 1000 times more sensitive than microscopy, on a series of samples from Dielmo, a holoendemic area of Senegal. Two-thirds of the microscopically negative individuals were found to harbour subpatent levels of *Plasmodium falciparum*, suggesting that more than 90% of the exposed population at any one time, i.e. in a cross-sectional survey, are chronically infected. This also means that the range of parasite loads harboured by humans with various degrees of exposure is remarkably large, probably reflecting a large range of effectiveness of the defence mechanisms against malaria parasites, none of which is fully efficient.

Keywords: malaria, *Plasmodium falciparum*, subpatent parasitaemia, Senegal

Introduction

Microscopical examination of the blood remains the main method for detecting malaria parasites. This technique is time consuming, difficult for technicians having only occasional practice, and there are indications that it is not very sensitive. This lack of sensitivity is rarely a matter of concern since it seldom impedes the diagnosis of acute attacks; nevertheless, it may influence our understanding of host–parasite interactions in endemic areas. The quantitative deficit of microscopy can be illustrated by several examples: detection of drug resistance at the RI level requires keeping patients under surveillance for 28 d, because parasite loads may be subpatent after their initial disappearance. Blood examination is also unable to detect all infected blood donors: one survey performed by microscopy during 4 years did not lead to the rejection of any of the 114 000 donors screened, while 112 recipients were infected (TIBURSKAYA et al., 1965; LUPASCU et al., 1967). In endemic areas, when cross-sectional surveys in which routine thick blood film examinations are carried out, it is known that examination of 200 microscope fields can detect only a subset of the parasite carriers. When 600 and 1000 fields were examined, prevalence in one area was found to increase from 41% to 61% and 70% respectively (DOWLING & SHUTE, 1965), indicating the prevalence of low-grade parasite carriers undetected by standard techniques (MOLINEAUX & GRAMICCIA, 1980; TRAPE, 1985). Longitudinal studies, in which blood films were repeated daily for one year, have shown that at least one positive film will be obtained from nearly all the exposed subjects (BRUCE-CHWATT, 1963). This indicates that all individuals are susceptible to malarial infection, whatever their age and level of acquired immunity. One drawback is that these results do not permit the distinction between (i) long-term persistence of chronic low-grade parasitaemia, fluctuating and occasionally reaching the microscopically detectable level, and (ii) a brief period of blood infection, after which parasites are eliminated.

There are therefore several situations in which more sensitive assays would be useful. Many new techniques have been proposed in recent years based on parasite visualization (SPIELMAN et al., 1988; RICKMAN et al., 1989; KAWAMOTO, 1991; ANTHONY et al., 1992; FERREIRA & FERREIRA, 1992; LEVINE & WARDLAW, 1992), detection of antigen (FORTIER et al., 1987; KHUSMITH et al., 1988; BEADLE et al., 1994), deoxyribonucleic acid (DNA) (FRANZEN et al., 1984; McLAUGHLIN et al., 1985; BARKER et al., 1986; ENEA, 1986; HOLMBERT et al., 1987; ZOLG et al., 1987; LANAR et al., 1989; KORPELA et al., 1992), or ribonucleic acid (WATERS & McCUTCHAN,

1989; WATT et al., 1992), and amplification of DNA by the polymerase chain reaction (PCR) (SETHABUTR et al., 1992; SNOUNOU et al., 1993a, 1993b).

Of these, the PCR appears to have the greatest sensitivity and, despite its limitations, to be of potential interest, i.e. for reassessing the host–parasite relationship. In preliminary assays we first selected sensitive PCR primers. When used on samples from individuals living under continuous exposure to parasite inoculation by mosquitoes, the PCR revealed a high prevalence of low-grade parasite carriers and therefore gave support to the idea that malarial infection is even more chronic than was previously thought.

Materials and Methods

Parasite strains cultured in vitro

For initial assays to determine the sensitivity level of PCR, 4 strains of *Plasmodium falciparum* (NF54, Palo Alto and FCIP 150 from Africa and D from Thailand) were cultured under standard conditions. Starting from a parasitaemia adjusted to 1% and 50% haematocrit, a range of parasite concentrations diluted with blood from healthy individuals containing uninfected red blood cells (RBC), leucocytes, platelets and plasma was prepared. Ten-fold serial dilutions were made to obtain final parasite densities ranging from one infected RBC (IRBC) per 10^2 RBC up to one IRBC/10^{10} RBC, in a final volume of 500 µL of whole blood. Assuming 2.5×10^9 RBC in 500 µL of whole blood at 50% hematocrit, there were theoretically 2·5 IRBC in each tube at 1/10^9 and 0·25 IRBC per tube in the 1/10^{10} dilution.

Isolates from patients

For PCR sensitivity assays, blood from 4 Africans and 2 Caucasians infected with *P. falciparum* was collected (plus heparin), parasitaemia was adjusted to 0·1% (one IRBC/10^3 RBC) by dilution in whole blood, and the same range of parasite concentrations (from 1 IRBC/10^3 RBC to 1 IRBC/10^{10} RBC) was prepared from each donor as described above, i.e. in whole blood containing leucocytes, etc.

Field studies were performed in the village of Dielmo in south Senegal, a malaria holoendemic area, in which the prevalence of *P. falciparum* carriers determined by thick blood film microscopy was 86% in children aged 1–14 years and 33% in adults over 60 years old (TRAPE et al., 1994). Thick blood films were prepared from each of the 247 individuals living in the village at the time. In addition, thick films had been made twice weekly from each individual over a period of 4 months preceding and following blood sampling. Each film was examined (200 fields, equivalent to c. 0·5 µL of blood) by 2 microscopists as described by TRAPE (1985). Finger-tip blood was collected in special heparinized capillary tubes of 500 µL

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capacity (custom-made at the Pasteur Institute), dispensed in cryotubes and frozen immediately, in the village, in liquid nitrogen. Samples were transported on dry ice to our laboratory and, after thawing, 100 µL of whole blood were processed for PCR. One-third of the extracted DNA was used for the PCR. The minimum detection level in these 'field' samples was theoretically about one IRBC in 10^8 RBC.

We selected 3 categories of cases among the 247 individuals studied. (i) Positive controls: 23 individuals whose thick films were positive at concentrations ranging from one IRBC/50 RBC to one IRBC/10^5 RBC. (ii) Negative controls: 15 persons who were sampled one to 2 weeks after completing a course of quinine treatment (25 mg/kg/d for 3 d) and whose blood films were negative and remained so at subsequent examinations. (iii) A test group of 21 individuals whose blood films were negative at the time of sampling and had been so (in the absence of drug treatment) for the last month. With 10 of these individuals, thick films remained negative during the further 2 months of follow-up. With the remaining 11, the thick blood films became positive again, more or less rapidly, within the next 2 months.

DNA extraction

For PCR sensitivity assays the entire whole blood content of the 500 µL tube, and for epidemiological studies 100 µL of whole blood, were processed using a standard phenol–chloroform procedure. Negative controls, DNA extracts from the blood of 6 healthy European individuals, were processed simultaneously with the test blood. 900 µL of distilled water were added to 100 µL of thawed whole blood to lyse the infected erythrocytes, and the lysate was vortexed and centrifuged for 5 min at 15 000 rev/min. The supernatant was discarded and the pellet was washed twice with 500 µL of distilled water. To the pellet were added 250 µL of TNE buffer (10 mM Tris, pH 8; 1 mM ethylenediaminetetraacetic acid (EDTA); 0·15 M NaCl) supplemented with 0·5% Triton X100®, 0·5% sodium dodecyl sulphate (10% solution), 5 mg/mL proteinase K, and 50 ng of herring DNA, and the mixture was incubated for one hour at 37°C. Following phenol–chloroform extraction (1/1, v/v) and centrifugation at 13 000 rev/min for 5 min, 10% ammonium acetate (3M) was added to the supernatant. Two volumes of ethanol were then added and precipitation allowed to proceed overnight at -20°C. The tube was centrifuged at 13 000 rev/min at 4°C for 30 min, the aqueous phase removed, one volume of cold 70% ethanol added to the pellet, and the tube was again centrifuged as described above. The pellet was dried at 80°C for 5 min and resuspended in 25 µL of TE buffer at pH8 (10 mM Tris-HCl, pH 7·2; 2 mM EDTA).

Other extraction methods were tested, including the method of KIMURA *et al.* (1990), distilled water, silica absorption and the Genomix® procedure; none proved sensitive enough.

Polymerase chain reaction

A 7 µL sample of the 25 µL of extracted DNA was subjected to PCR using standard conditions, 2 units of *Taq* polymerase (Promega) and 1 µM of the chosen oligonucleotide primers in a total volume of 100 µL. The amplification was performed in a Hybaid® reactor using the following cycle: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for 40 cycles followed by 1 cycle at 72°C for 5 min.

Controls consisted of *P. falciparum* DNA (100 ng), DNA from the gene corresponding to the primers used, DNA extracted by the same procedure from control uninfected individuals, and buffer only.

Following preliminary assays, all extraction and PCR procedures were performed under sterile conditions, in a laboratory where malarial DNA was not usually handled, using Gilson pipettes equipped with filter tips (Art-tips®, Molecular Bio Products, San Diego, Califor-

nia, USA) to avoid pipette contamination. Analysis of PCR products was made by agarose gel electrophoresis (4% NuSieve® agarose) in ethidium bromide solution (0·5 mg/mL) and the amplified products were visualized under ultraviolet (u.v.) light. The gel was then blotted on to nylon membranes (Hybond®), hybridization was performed using an internal oligonucleotide, from the corresponding clone, radiolabelled with ^{32}P (SAMBROOK *et al.*, 1989), and the membranes were auto-radiographed.

Primers

Following preliminary studies using primers previously employed in PCR studies (such as MSA1, MSA2, CS, TRAP) (DAUBERSIES *et al.*, 1994) and others derived from new antigens studied in our laboratory, we chose the following 3 pairs of primers for the study: one derived from a novel merozoite surface antigen of *P. falciparum* named MSP3 (OEUVRAY *et al.*, 1994) (5´CAT-GAAAGGGCAAAAAATGCTT3´), (5´GGAAAATATA-TCTAAGGAAAATGAG3´), one from the sporozoite and liver stage antigen of *P. falciparum* (SALSA) (Bottius *et al.*, paper submitted for publication) (5´CGAGTAAG-TACTAGTGATACTCCTGG3´), (5´CTTTGGAGA CTT-TTCTAGAACCTTTTCTTG3´) and one clone (DG 157) from a gene in our genomic library of *P. falciparum* DNA (5´AGTGATGAACTTTTTAATGAATTATTAAA3´), (5´TG-TTGTTCTTGTTGAACACTTTTTAC TAA3´).

Preliminary results, using DNA from 3 cultured strains and 3 patient isolates, showed for each of them that (i) the corresponding gene was present in all strains, (ii) PCR consistently yielded a single band of amplified products, (iii) this band showed no size polymorphism, and (iv) these pairs of primers could detect very low parasite concentrations.

Results

The level of detection by the PCR and the reproducibility of results were evaluated first using synchronous cultures at the ring trophozoite stage from 4 strains in over 20 separate experiments. The lowest level detected was between one IRBC/10^8 RBC and one IRBC/10^9 RBC with each of the strains, whilst test dilutions containing 1 IRBC/10^{10} RBC all gave negative results, using amplified material and examination under u.v. light (Fig. 1). Hybridization with radioactive probes improved the de-
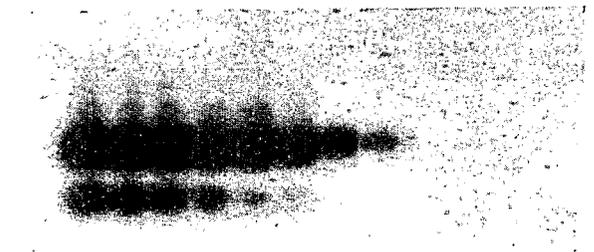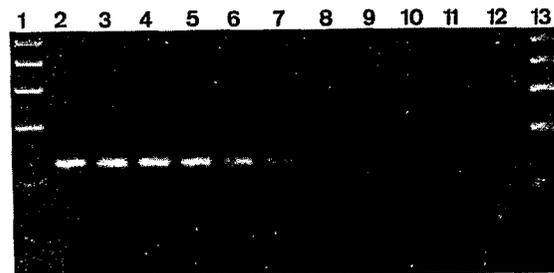

Fig. 1. Level of detection of malaria parasites obtained by PCR using the primers derived from DG157 and DNA extracts from synchronous cultures of *P. falciparum* at ring trophozoite stage with parasite concentrations from one infected erythrocyte in 10^2 cells (lane 2) to one in 10^{10} (lane 10). Lanes 1 and 13, molecular mass 'ladders'; lanes 11 and 12, negative controls (extracts from uninfected erythrocytes). Upper panel: ethidium bromide staining. Lower panel: after hybridization, on Hybond® with an internal radiolabelled oligonucleotide probe.

tection level: samples previously positive at $1/10^8$ became positive at $1/10^9$, but the $1/10^{10}$ dilutions remained negative. The variation between experiments was low: for example, using 5 different sets of extracts from strain D (from 5 different cultures) and SALSA primers, one IRBC/10^9 RBC could be detected in 3 extracts and one IRBC/10^8 RBC in 2 extracts.

Additional assays aimed at evaluating the detection level used blood from patients infected with *P. falciparum*, which therefore contained only young ring forms. Levels of one IRBC/10^8 RBC and one IRBC/10^9 were again detected using u.v. light. (Fig. 2). With these isolates, as with the cultured strains, the most sensitive

N°	Age (years)	Results from successive thick smears	PCR
793	60	- - - - - - - ⊙ - - - - - - - - - - -	+
813	5	- - - - - - ⊙ - - - - - - - - - - - -	+
726	5	- - - - - - - - - - ⊙ - - - - - - - -	+
835	5	- - - - - - - - - - ⊙ - - - - - - - -	-
528	17	- - - - - - - - ⊙ + + + + - - - - - +	-
557	1	- - - - - - ⊙ - - - - - - - - + + +	-
723	1	- - - - - - - ⊙ - - - - - - + + + + +	+
1305	2	- - - - ⊙ - - - - - - - + + - - - - -	+
570	42	- - - - - - - ⊙ - - - - + - + + + + +	-

Fig. 3. Detailed results from 9 cases (5 malaria-positive and 4 negative by PCR) from the test series in Senegal. The results from 19 thick blood films made during 10 weeks from each subject are shown; – = negative, + = positive by microscopy. Circled samples were used for DNA extraction and further amplification.

films. In contrast, with subject no. 528 the negative thick film made at the same time as the PCR sample was collected was confirmed as negative by the PCR, although the next 4 thick films were positive. Positive PCR results were no more frequently obtained with subjects who became positive by thick film sooner or later during follow-up than in those who remained uniformly negative (Fig. 3). Thus, the low-grade parasitaemia detected by PCR had no predictive value; it did not signify that parasitaemia was rising and would reach microscopically detectable levels in the following days.

Cases in which the PCR was positive and the thick film negative occurred at least as frequently in older patients as in the younger ones—in 8 of 10 subjects aged more than 25 years (2 being 60 and 70 years respectively) and 3 of 7 aged less than 5 years.

Table. Results of PCR amplification of samples from subjects in Senegal with a history of a long series of malaria-negative thick blood films

Age group	No. studied	PCR results[a]		
		MSP3	SALSA	DG157
Months				
11–18	4	2	2 (2)	3 (3)
Years				
4–5	3	0	0 (0)	1 (1)
10–17	4	2	1 (2)	2 (2)
25–40	4	2	4 (4)	4 (4)
40–70	6	1	1 (3)	4 (4)
Totals	21	7	8 (11)	14 (14)

[a]No. of cases with *P. falciparum* diagnosed by visualization of the amplified band in agarose gels under u.v. light and (in parentheses) after probing with an internal radiolabelled oligonucleotide probe.
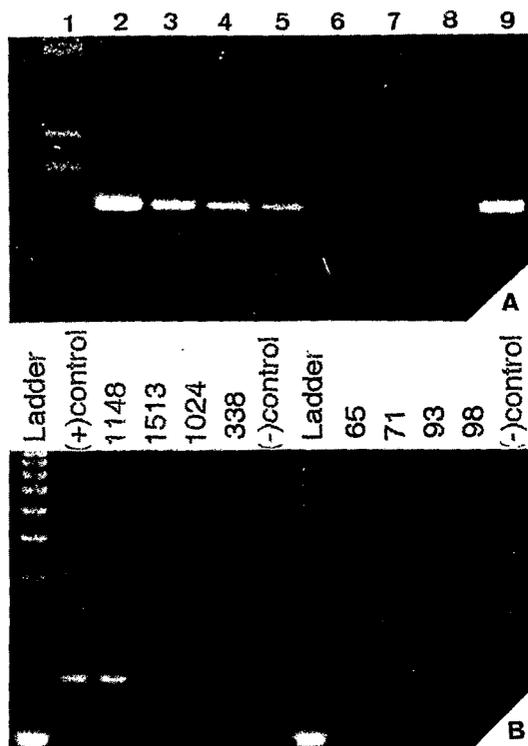

Fig. 2. (A) PCR amplifications from a range of dilutions of patients' malaria-infected erythrocytes mixed with normal erythrocytes (lanes 2, $1/10^4$; 3, $1/10^5$; 4, $1/10^6$; 5, $1/10^7$; 6, $1/10^8$; 7, $1/10^9$); lanes 8 and 9: negative and positive controls. (B) The results obtained with some of the individuals studied in the Senegalese village of Dielmo (5 PCR positive cases [nos. 1148, 1513, 1024, 338, 65] and 3 negative cases [71, 93, 98]).

and reliable results were obtained with primers derived from DG 157; the levels of detection reached were 10 to 100 times better than with primers corresponding to polymorphic regions of MSA1 and MSA2 (DAUBERSIES et al., 1994) (results not shown).

Since the levels of detection by PCR proved 100 to 1000 times better than those achieved with thick blood films, this technique was then applied to blood samples from individuals living in the endemic area of Dielmo.

Twenty-two of the 23 positive control subjects were positive by PCR with each of 3 pairs of primers; the remaining individual gave a positive result only after a second extract had been prepared.

All 15 negative control subjects gave negative PCR results with the 3 primers. All these experiments were done in triplicate, with known positive and negative controls included each time.

A representative example of the diverse results obtained with the 21 individuals in the test group is presented in Fig. 3. Subjects no. 793, 813 and 726 gave a positive PCR result despite consistently negative thick

In total, 14 of these 21 subjects were positive by PCR, i.e. having a very low level parasitaemia. Different primer pairs had different sensitivities (Table), but the results were convergent, i.e. subjects giving positive results with the less sensitive primers MSP3 and SALSA were also positive with DG157. Hybridization with internal probes slightly increased the sensitivity (Table).

The reproducibility of the assay from experiment to experiment was satisfactory: for example, the 21 extracts from the test group were amplified twice by PCR and yielded exactly the same results with each of the 3 pairs of primers used. Three RBC pellets from the same series, and 2 negative controls, were subjected 'blind' to another extraction and produced exactly the same pattern of results with each set of primers.

Discussion

Our results confirm that PCR, when employed with optimal primers, can detect *P. falciparum* parasitaemias at least 100 to 1000 times lower than microscopical examination can. The technique revealed a high frequency of very low-grade parasite carriers amongst individuals living in a holoendemic area who had negative thick blood films.

The gain in sensitivity achieved by PCR was obvious even when compared to the performance of well-trained, experienced technicians, who can routinely detect one parasite in 2×10^6 RBC by examining 200 fields of a thick film (BRUCE-CHWATT, 1985; TRAPE, 1985), and much more so when compared with technicians having only occasional experience of blood film examination. The levels achieved were sufficient for the purpose of this study, yet they could be improved if needed either by hybridizing the amplified products with a labelled probe, or by making extracts from larger amounts of blood than the 100 μL we used. Although we did encounter accidental DNA contamination (particularly in preliminary studies, before changing to a dedicated laboratory for all extraction and amplification steps), the reliability of these results can be considered satisfactory. This was demonstrated by (i) the negative controls included in the sensitive assays derived from distinct individuals and processed at the same time as the tests; (ii) the negative results obtained with dilutions of one IRBC in 10^{10} RBC, corresponding to less than one parasite per tube; (iii) results obtained with the 2 groups of positive and negative controls from the endemic area; and (iv) the convergence of results obtained with the 3 sets of primers in the test group from the same area.

The relatively high prevalence of positive PCR results among samples from subjects with negative thick blood films was particularly impressive and epidemiologically significant in view of the stringent selection of the cases we studied. We did not study cases whose thick films were alternately positive and negative, indicating a fluctuating parasitaemia, but we chose to study samples taken from patients with a prolonged history of negative films, whether or not they subsequently became positive. These were the most significant cases for evaluating the prevalence of submicroscopic parasitaemia and the potential of the technique. Thus, even taking into account the small number of individuals studied, it is impressive that such a high proportion of carriers of very low-grade parasitaemia was detected.

Previous longitudinal studies in which blood film examinations were repeated had indicated that the cumulative prevalence of parasite carriers over a year was close to 100% (BRUCE-CHWATT, 1963; PETERSEN *et al.*, 1990) and this was confirmed in the village that we studied, since blood films examined twice a week revealed that 98·6% of the villagers harboured *P. falciparum* at least once during 4 months' follow-up (TRAPE *et al.*, 1994). The results obtained by PCR were in keeping with these observations.

In addition, the PCR results indicate that the parasitaemia in these individuals was not intermittent, as suggested by microscopy, but continuous; the parasite carrier state is chronic in most individuals. In the village studied the prevalence in adults over 20 years of age was 53% by slide examination in a cross-sectional survey, and 80% in the children (TRAPE *et al.*, 1994). Prevalence by PCR, in 100 μL blood samples, was 8/10 in the adults with negative blood films, close to the 80% detected in children in the same village. The true prevalence in the adult population was therefore probably at least 90% at any one given time. These figures, albeit surprisingly high, are in agreement with previous mathematical models of epidemiological data (DIETZ *et al.*, 1974; MOLINEAUX & GRAMICCIA, 1980).

Furthermore, when one takes into account the sequestration of *P. falciparum* in deep-seated capillaries, the total parasite load harboured by PCR-positive but micro-

scopically negative individuals cannot be as low as it would seem at first glance. Our results provide an explanation for the findings of MUIRHEAD-THOMSON (1954), that mosquitoes could be infected when fed on individuals with no microscopically detectable gametocytes. Another potential consequence is the occurrence of clinical attacks of malaria, without reinfection, if there is a temporary decrease in the defence mechanisms which maintain parasitaemia at low levels.

In non-immune patients with malaria, the parasitaemia usually ranges from about one to 600 parasites per 1000 RBC (BRASSEUR *et al.*, 1990). In partially or fully immune patients it was found as low as one per 10^5 to one per 10^6 RBC, the lower limit of sensitivity of thick blood films examined even in the best conditions. PCR now suggests that there is no clear limit, and that parasitaemias at least in the range of one parasite per 10^6 to 10^8 RBC are frequent in immune adults. As a consequence, our perception of the overall picture of the host–parasite interaction is considerably modified. The range of parasite loads harboured by humans with various degrees of immunity now appears to be extraordinarily wide. This probably reflects a large number of genetically and immunologically dependent interactions between host and parasite. It appears that none of the host's defence mechanisms, even those resulting from the very frequent challenge in the holoendemic area that we studied (TRAPE *et al.*, 1994), can achieve sterile immunity even for a short time. In the light of those results, malaria appears even more than before as a chronic disease, a finding which has obvious implications for epidemiology and for vaccine development.

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