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We report on an analysis of the constraints of PCR typing of field *Plasmodium falciparum* isolates by using a few highly polymorphic markers, MSA-1, MSA-2, TRAP, and CS. We show that the reactions are specific for the *P. falciparum* species. The detection threshold (minimum number of parasites required to detect a visible band by ethidium bromide) differed from one marker to the other and, within one locus, from one primer combination to the other. Importantly, the various MSA-1 and MSA-2 reference alleles were amplified with the same efficiency. Amplification from reconstituted allele mixtures indicated that at certain allele ratios, the most abundant allele interfered with the amplification of the less abundant one. An analysis of nine isolates collected from patients with acute malaria in Dielmo, Senegal, during a transmission season when the inoculation rate was one infective bite every second night is presented and discussed. All samples contained more than one parasite type. A significant polymorphism was observed for the four markers. Novel *Taq*I restriction fragment length polymorphisms were found for the TRAP gene, and TRAP gene typing alone allowed a distinction between the various isolates. MSA-1 and MSA-2 gave multiple band patterns specific for each sample.

While it is now well established that *Plasmodium falciparum* malaria parasites are polymorphic (for a review, see reference 14), the extent of genetic diversity of local parasite populations and the role that polymorphism plays in the interactions with both the *Anopheline* and the human hosts remain largely unknown. The epidemiological survey undertaken in the village of Dielmo, in Senegal, where malaria is holoendemic (39), provides a unique opportunity to address these issues, because a large number of parameters influencing the host-parasite relationships, including human immune responses, were investigated. We decided to type the parasites circulating in this village to analyze the extent of genetic diversity of the local parasite population and to follow the fates of specific parasites in a longitudinal analysis of infected individuals.

The methods presently used to type field isolates present inherent limitations. Isoenzyme typing (2, 4, 8) requires substantial amounts of parasites, restricting the analysis to samples with high parasite densities or requiring short-term cultivation of samples presenting with low levels of parasitemia. Monoclonal antibody typing requires parasites at a particular stage of development, necessitating short-term maturation of blood-stage parasites (5, 6, 20). The analysis is done with a battery of reagents that define distinct serotypes, but it is limited by the incapacity to type novel serotypes possibly present in field isolates. An alternative to these methods is the use of the sensitive tool that amplification by PCR represents (30). PCR has the major advantage of eliminating the need for in vitro manipulation of parasites, because DNAs from circulating ring-stage parasites can be used to analyze a large number of genetic loci, including those expressed at a different stage or in the mosquito vector. Furthermore, PCR generates the material to be typed, instead of consuming it, and once a gene fragment has been amplified, analysis of the fragment can be

performed by various methods, investigating distinct polymorphic characters, such as size, the presence and distribution of restriction sites, allellic type, or DNA sequence. If during this process new alleles are discovered, the relevant PCR products can be readily incorporated into the battery of typing probes.

To date, PCR has been successfully used as a highly sensitive diagnostic method (3, 28, 31, 32, 36, 38, 40, 41) and for investigating the genetic diversity of specific loci coding for major vaccine candidates such as the MSA-1 (15, 22, 34, 37), MSA-2 (18, 19, 22, 34), or CS (1, 17) protein or for the highly polymorphic S antigen (16). The use of PCR for typing purposes necessitates that the analysis be standardized to provide the quantitative and qualitative framework necessary to describe the parasites present in biological samples. This is necessary for investigating the structure of parasite populations and comparing samples, whether they are collected from the same individual (e.g., in the case of recrudescences or in the longitudinal analysis of infections) or from specific geographical locations or regions.

The work reported here describes the results obtained in investigating some methodological aspects of a PCR-based typing strategy and outlines the constraints limiting interpretation of the data. A few highly polymorphic loci, MSA-1, MSA-2, TRAP, and CS, have been chosen to devise PCRs to rapidly differentiate parasite isolates from small volumes of blood samples. These markers differ in the type of polymorphisms that they present. Briefly, a large number of MSA-1 alleles, a gene coding for a merozoite surface antigen of about 200 kDa, have been sequenced. The various alleles can all be grouped into two major allelic families (24, 37). The gene contains several distinct regions that differ in their degrees of conservation. Region 2 is particularly polymorphic, and three distinct allelic families have been described, MAD20, K1, and RO33 (15, 22, 28, 37). The gene encoding the second merozoite surface antigen, MSA-2, is organized into two external highly conserved regions (11, 33) flanking a polymorphic central domain. This polymorphic region contains unique sequences that belong to two distinct families, 3D7 and FC27, as well as a mosaic of family-specific repeated sequences that, within a family, vary in length, number, and organization (18, 22, 27, 34). The polymorphism of the CS gene is documented

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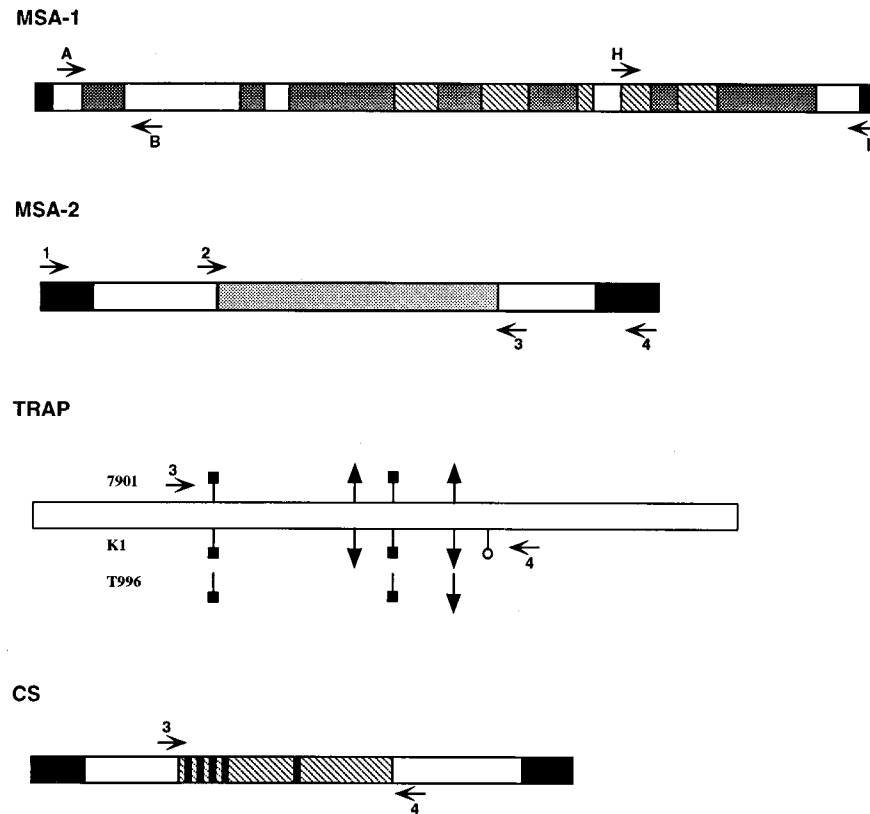
FIG. 1. Schematic maps of the genes used as typing markers showing the regions amplified and the positions of the primers used. For all genes, conserved regions are left unfilled, apart from external conserved regions coding for the N- and C-terminal hydrophobic sequences, which are depicted in black. The MSA-1 gene is shown as proposed by Tanabe et al. (37) and is divided into 17 discrete blocks presenting various degrees of homology. The shaded regions represent variable sequences, and the striped regions indicate semiconserved domains. The MSA-2 gene is reproduced from Marshall et al. (18, 19). The central variable region is dotted. A schematic restriction map of the TRAP gene in the region amplified with primers 3 and 4 is indicated. The position of the restriction sites is indicated for strain 7901 (alias FUP/SP Palo Alto), strain K1, and clone T9-96, which are representative of the three major alleles of TRAP as classified by Robson et al. (29), and indicate *Hin*fI, *Taq*I, and *Bgl*II, respectively. The central region of the CS gene encoding the four amino acid repeats (9) is represented as hatches. The variant repeat (encoding NVDP) is indicated in black.

for the diverse unique regions encoding T epitopes (17, 26) and for the central domain of the gene, which encodes two types of four-amino-acid repeats that vary in number and relative position from one isolate to the other (21). The TRAP gene consists of unique sequences that show limited restriction site polymorphism (29). Our results indicate an extensive genetic polymorphism of the four loci in isolates from Dielmo and, furthermore, point to an important complexity of the parasites circulating in the peripheral blood.

MATERIALS AND METHODS

Parasite isolates and culture conditions for reference lines. The FUP/CB Palo Alto strain was cultivated as described by Fandeur et al. (10), and highly synchronous parasites were obtained by plasma gel flotation and then sorbitol lysis a few hours after invasion for two successive cycles. Reference lines were clone 89F5 from the FUP/SP Palo Alto strain (10), clone ItG2G1 (13), and the Senegalese isolates 2, 4, and 7 (22). The wild isolates studied here were from blood samples collected in the Dielmo village (39). All were from patients with acute symptomatic malaria episodes and were collected during June to August 1990. DNA samples 1 to 9 were extracted from blood samples presenting 4.6, 0.6, 0.8, 0.8, 4, 1.7, 0.9, 2.2, and 1.2% parasitemias, respectively. Blood was recovered by venipuncture under citrate. After centrifugation and recovery of the plasma, the erythrocyte pellet was frozen in liquid nitrogen. After transportation, it was stored at −20°C.

Plasmodium vivax and *Plasmodium ovale* DNAs were kind gifts of P. David and P. Daubersies, respectively.

Plasmodium malariae parasite DNA was extracted from a blood sample collected from a human with an acute infection in Cayenne, French Guiana.

DNA extraction. DNA was extracted by standard procedures. About 100 μl of erythrocytes was lysed by three freezing-thawing cycles. Free parasites were washed three times with distilled water and were resuspended in 5 volumes of TEN buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA, 0.15 M NaCl, 0.5% Triton X-100, 0.5% sodium dodecyl sulfate) and 5 mg of proteinase K per ml. After 1 h of incubation at 37°C, the DNA was extracted twice with 1 volume of phenol-chloroform-isoamyl alcohol (25/24/1) and was precipitated with ethanol in the presence of 0.3 M sodium acetate (pH 5.5). The DNA was resuspended in 50 μl of H$_2$O.

Sequences of the primers used. Figure 1 shows a schematic map of the genes studied and the regions amplified with the specific primers. The primers used for the MSA-1 gene were MSA-1 A (5′-AAG CTT TAG AAG ATG CAG TAT TGA C-3′) and MSA-1 B (5′-ATT CAT TAA TTT CTT CAT ATC CAT C-3′) as well as MSA-1 H (5′-AAT ATT ATA ATG GTG AAT CAT CTC C-3′) and MSA-1 I (5′-AGT ATT AAT AAG AAT GAT ATT CCT A-3′). For the MSA-2 gene the following primers were used: MSA-2 1 (5′-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3′) and MSA-2 2 (5′-AAC GAA TTC ATA AAC AAT GCT TAT AAT ATG AGT-3′) as well as MSA-2 3 (5′-GAT GAA TTC TAG AAC CAT GCA TAT GTC CAT GTT-3′) and MSA-2 4 (5′-ATA TGG CAA AAG ATA AAA CAA GTG TTG CTG-3′). For the amplification of the TRAP gene, the following primers were used: TRAP 3 (5′-ATG TAA CTT GTA TGC TGA TTC TGC ATG G-3′) and TRAP 4 (5′-TAT CTT CAC TAT TAG GTA CGT GCC TAT TTC C-3′). The sequence of the primers used to amplify the central region of the CS gene were 5′AGA GAT GGA AAT AAC GAA GAC AAC GAG-3′ (CS 3) and 5′-GTC ATT TGG CAT TAA GTG ACC TTG TCC-3′ (CS 4).

Amplification reaction. A total of 1 to 5 μl of DNA was amplified in a final volume of 50 μl in the presence of 200 μM (each) deoxynucleoside triphosphate, 1 μM (each) primer, 2.5 U of *Taq*I DNA polymerase (Promega, Charbonnières, France) in the buffer supplied by Promega (12 mM MgCl$_2$). The reactions were performed in a Hybaid thermocycler. The standard reactions parameters were 15 s at 94°C, 2 min at 55°C, and 2 min at 72°C for 1 cycle; 2 s at 94°C, 2 min at 55°C,

TABLE 1. Characteristics of reference clones or isolates used in the study

Parasite DNA	Allelic type used as reference for:		
	MSA-1 A–MSA-1 B	MSA-1 H–MSA-1 I	MSA-2 2–MSA-2 3
Clone 89F5	K1	MAD20	INDO/3D7
Clone ItG2G1	MAD20	K1	INDO/3D7
Senegal 2	RO33		
Senegal 4			FC27

and 2 min at 72°C for 35 cycles; and 2 s at 94°C, 2 min at 55°C, and 10 min at 72°C for 1 cycle.

Analysis of PCR products. Analysis of the genetic diversity of the amplified products was carried out in several steps. Size polymorphism was investigated for the four loci by loading 5-μl aliquots of the PCR mixtures onto agarose gels (SeaKem GTG; Tebu, Le Perray en Yvelines, France) in TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.01 M EDTA [pH 8.0]) in the presence of 0.5 μg of ethidium bromide per ml.

Identification of TRAP allelic types by restriction fragment length polymorphism (RFLP) analysis was done by incubating 1 to 2 μl of the amplification reaction with the TRAP 3–TRAP 4 primer pair with 2 to 5 U of *Taq*I restriction enzyme (Biolabs, Montigny-le-Bretonneux, France) under the conditions recommended by the supplier for 1 h and analyzing the product on a 1.5% agarose gel (SeaKem GTG; Tebu).

Assignment of MSA-1 and MSA-2 to specific allelic families was done by hybridizing Southern blots with allele-specific probes (22). Probes were prepared by nick translation, with a nick translation kit from Boehringer Mannheim (Meylan, France), of fragments amplified from reference clones or isolates whose sequences have been determined (Table 1). Alternatively, the recombinant plasmids obtained after cloning the various MSA-1 and MSA-2 reference probes (TA cloning kit; In Vitro Gene, Oxon, United Kingdom) were used in the nick translation reaction. Unincorporated nucleotides were eliminated by spin dialysis. Hybridization was done as described previously (22), and the blots were washed under increasing stringency (6× SSC, 2× SSC, 0.5× SSC, and 0.1× SSC at 65°C [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]). At high stringency, the probes hybridize specifically to the genes belonging to the same family.

RESULTS

Choice of primers and amplification conditions. A schematic map of the genes studied is presented in Fig. 1. The primers chosen, derived from sequences conserved in all alleles sequenced to date (World Health Organization database), flank polymorphic regions. The optimal conditions for ampli-

fication of each marker were analyzed with the DNA from 89F5, a clone derived from the monkey-adapted FUP/SP Palo Alto isolate (10). Several parameters (primer concentration, concentration of salts, temperatures of annealing and extension, source of enzyme) have been studied (data not shown). This resulted in the definition of a convenient method, described in detail in Materials and Methods, that allowed amplification for all markers under the same experimental conditions.

Specificities of the PCRs. In many areas where malaria is endemic, and in particular in the village of Dielmo (39), several *Plasmodium* species are in circulation. We therefore examined whether the primers and PCR conditions chosen were specific for the *P. falciparum* species. We prepared a series of PCRs mixtures in which DNAs from *P. falciparum*, *P. malariae*, *P. ovale*, and *P. vivax* parasites were used with the primers MSA-1 A–MSA-1 B, MSA-1 H–MSA-1 I, MSA-2 2–MSA-2 3, MSA-2 1–MSA-2 4, TRAP, 3–TRAP 4, and CS 3–CS 4. The results are shown in Fig. 2. For all reactions, amplification was restricted to *P. falciparum* DNA. This was also observed for the reaction with CS 3–CS 4 (data not shown). This indicated that the reactions with *P. falciparum*-derived primers were species specific. The quality of the DNAs from *P. malariae* and *P. ovale* was verified by using the species-specific primers RPM 1–RPM 2 and RP O1–RP L6, respectively, as described by Snounou et al. (35). Amplification from *P. vivax* was obtained by using the *P. vivax*-specific primers RBP R–RBP F (12). In each case, the predicted band was observed (Fig. 2, as indicated). No amplification was observed with any of the primers tested with human DNA or *Saimiri sciureus* monkey DNA (data not shown). This is consistent with the absence of amplification in the *P. malariae*, *P. ovale*, and *P. vivax* samples, which contained significant amounts of contaminating human DNA (Fig. 2).

Sensitivity. The threshold of detection for the various markers and primer combinations was investigated by performing the reactions with serial dilutions of DNA from clone 89F5. The same batch of DNA was used for all reactions. After amplification under the standard conditions established as described above, the PCR products were analyzed on agarose gels and were detected by ethidium bromide staining, which, within a certain range, gives a good estimate of the DNA
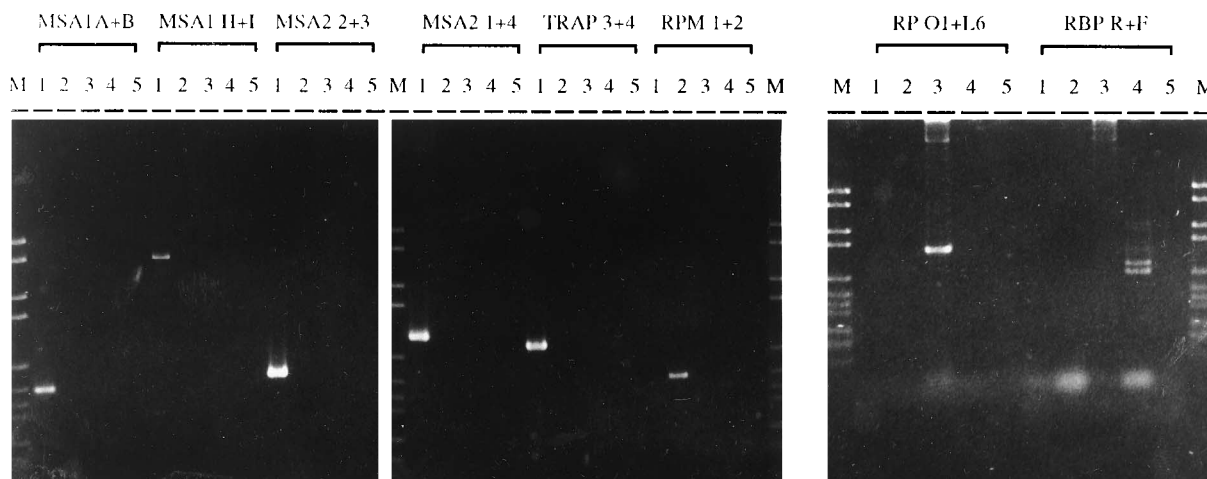

FIG. 2. Species specificity of the various amplification reactions. The PCR products obtained after amplification with the oligonucleotide primer pairs indicated above the lanes were analyzed on 1.5% agarose gels and were stained with ethidium bromide. MSA-1 A–MSA-1 B, MSA-1 H–MSA-1 I, MSA-2 2–MSA-2 3, MSA-2 1–MSA-2 4, and TRAP 3–TRAP 4 were derived from *P. falciparum* genes; RPM 1–RPM 2, RPO1–L6, and RBP R–RBP F were derived from *P. malariae*, *P. ovale* (35), and *P. vivax* (12), respectively. The genomic DNAs used in the reactions were from *P. falciparum* (lanes 1), *P. malariae* (lanes 2), *P. ovale* (lanes 3), and *P. vivax* (lanes 4). In lanes 5, no DNA was added to the reaction tubes.
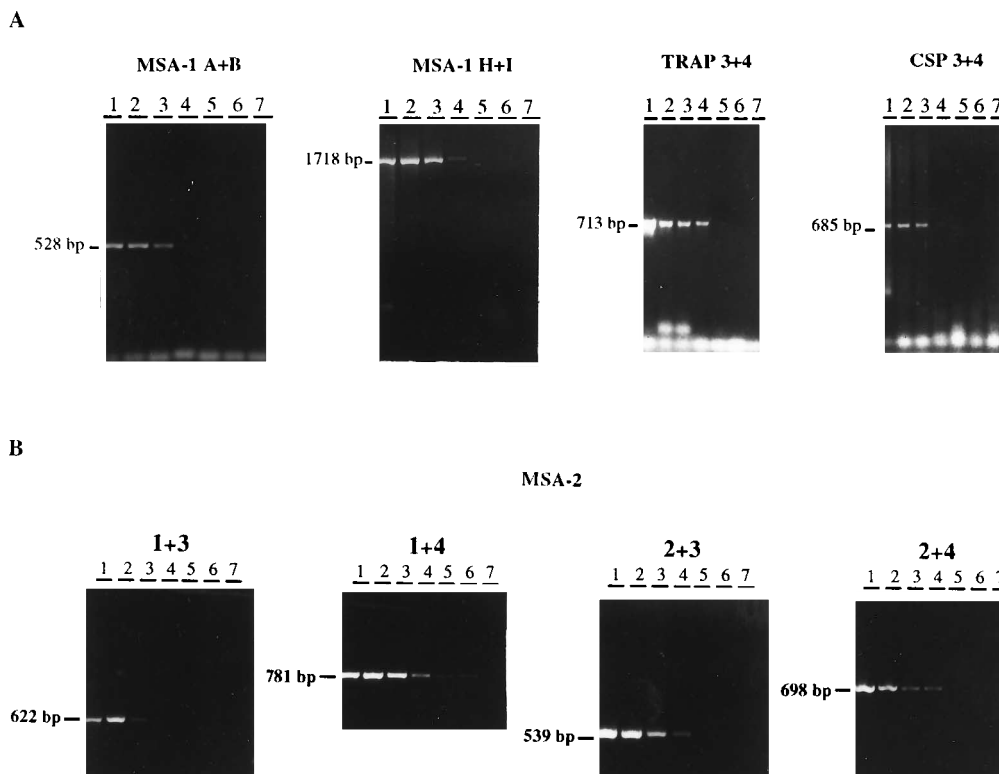
FIG. 3. Detection threshold of the various typing reactions. Serial 10-fold dilutions of the 89F5 clone were amplified with the primer combinations indicated in a final volume of 50 μl, and 5-μl aliquots were analyzed on 1.5% agarose gels and visualized by ethidium bromide staining. (A) The products of the MSA-1, CSP, and TRAP reactions were analyzed. (B) Various MSA-2 primer combinations were used. The quantities of DNA present in each tube were as follows for all reactions: 25 ng (lanes 1), 2.5 ng (lanes 2), 250 pg (lanes 3), 25 pg (lanes 4), 2.5 pg (lanes 5), 0.25 pg (lanes 6), no DNA added (lanes 7). The size of the fragment amplified in each reaction is indicated on the left of the gels.

concentrations. The results, shown in Fig. 3, indicate that the limiting dilution at which a visible PCR product was obtained varied for the different markers and depended on the primer combination used. The intensity of ethidium bromide staining roughly reflected the dilution of the master DNA preparation, indicating that the reactions used in the present study were semiquantitative. As shown in Fig. 3A, a minimum of 25 pg of DNA was required for a positive reaction with MSA-1 A–MSA-1 B and CS 3–CS 4, whereas it was 2.5 pg of DNA for MSA-1 H–MSA-1 I and TRAP 3–TRAP 4. Four primer combinations were tested for MSA-2 on serial dilutions of 89F5 DNA (Fig. 3B). The 1+4 combination was the most sensitive, detecting 0.25 pg of DNA, while the 2+3 and 2+4 combinations necessitated a minimum of 25 pg of DNA and the combination 1+3 necessitated 250 pg of DNA to generate a visible band. For the MSA-1 and MSA-2 loci, hybridization was used for assigning the alleles to a specific family (for example, see Fig. 6). Hybridization resulted in an increase in the detection sensitivity by at least a factor of 10 (data not shown).

The detection threshold was also estimated by using serial parasite dilutions in fresh blood, which produced a large range of parasite DNA concentrations in a fixed amount of potential contaminants and interfering factors. This is more relevant to the situation one faces when one is analyzing field samples presenting parasite densities distributed over a wide range. Parasites at the early ring stage were harvested from a highly synchronous culture and were serially diluted in freshly collected human blood, and DNAs were prepared from each dilution. Confirming our previous observations, the detection threshold again depended on the markers used, and it was

usually in the same range as that defined above. It differed in some cases: it was less sensitive for MSA-1 A–MSA-1 B and was more sensitive for MSA-2 2–MSA-2 3 (data not shown). One important factor that interfered with the reaction yield was the presence of human DNA. Indeed, when purified parasite DNA was amplified in the presence of various quantities of human DNA, the sensitivity of some reactions was decreased by a factor of 10 (data not shown).

We investigated the possibility of mixing primers for MSA-2 2–MSA-2 3, TRAP 3–TRAP 4, and CS 3–CS 4 in the same tube in an approach similar to that proposed by Wooden et al. (41). The various fragments were successfully amplified and were easily identified on agarose gel because they presented as bands of different size ranges that could be conveniently distinguished. However, the efficiency was reduced by a factor of 10 in comparison with the yield obtained with each primer alone (data not shown). This approach was therefore not pursued further.

Amplification of reference alleles. Using the same semiquantitative approach, we next investigated whether the various reference alleles were amplified with the same efficiency. This was done for MSA-1 and MSA-2 by comparing the intensity of ethidium bromide staining of the PCR products generated with serial dilutions of DNA prepared from the reference lines or clones listed in Table 1. For MSA-1 A–MSA-1 B, the three major allelic forms described, MAD20, K1, and RO33, were amplified with the same efficiency; i.e., bands of similar intensities were observed in reactions that used similar amounts of parasite DNA (data not shown). The detection threshold was identical for the three alleles. This was also observed for the
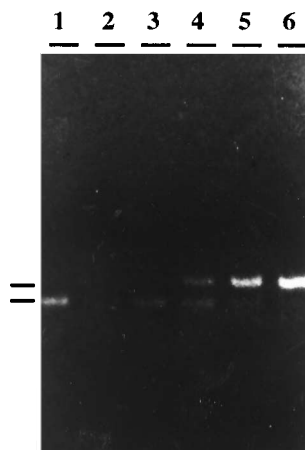
FIG. 4. MSA-1 A–MSA-1 B amplification from reconstitued allele mixtures. A constant amount (250 pg) of FUP/CB Palo Alto DNA was mixed with decreasing quantities of clone 89F5 DNA. After amplification, the products were analyzed on agarose gels and stained with ethidium bromide. The quantities of 89F5 DNA present were 0.25 pg (lane 1), 2.5 pg (lane 2), 25 pg (lane 3), 250 pg (lane 4), 2.5 ng (lane 5), 25 ng (lane 6). The smaller PCR product (about 430 bp) corresponds to the FUP/CB Palo Alto allele, whereas the larger one (about 528 bp) corresponds to the 89F5 allele (22).

two major allelic families of MSA-2, FC27, and INDO/3D7 (data not shown).

Amplification from allele mixtures. Because mixed infections are frequently observed in areas where malaria is endemic (5–8) we investigated semiquantitatively the amplification of each allele from mixtures reconstituted with different allele ratios. A matrix was prepared in which dilutions of DNA from one isolate (FUP/CB Palo Alto) were mixed with serial dilutions of DNA from another one (89F5). As shown in Table 1, both DNAs encode alleles that belong to distinct allelic families and that differ markedly in sequence. The FUP/CB Palo Alto allele migrates as an approximately 430-bp band and the 89F5 product migrates as a 528-bp band (22). Figure 4 illustrates one such series, obtained for the reaction with MSA-1 A–MSA-1 B with a fixed amount of FUP/CB Palo Alto DNA (250 pg) mixed with various quantities of 89F5 DNA. This shows that the reaction products reflect the relative proportions of both alleles, as long as this ratio was 1/1 (lane 4) or 1/10 (lane 5). When the ratios exceeded this range, the product corresponding to the least abundant allele was no longer visible. This occurred even when we used an amount in which the

allele would be detected if it was amplified alone (for the 89F5 allele, see lanes 2 and 3; for the FUP/CB Palo Alto allele, see lane 6). Similar results were observed for other fixed concentrations of FUP/CB Palo Alto DNA (2.5 or 25 ng). The same phenomenon of extinction by the most abundant allele was observed for the MSA-2 2–MSA-2 3 PCR with two alleles belonging to the same allelic family (INDO/3D7) (data not shown). These results indicate that the presence of multiple alleles (closely related alleles or more distant ones) in the DNA preparation influences the amplification yield of each allele when primers derived from common conserved regions are used, and therefore is likely to prevent amplification (and hence detection) of the parasites present in minor proportions.

Analysis of field samples. A series of nine samples collected from patients with acute malaria in Dielmo and kept as frozen dry blood cell pellets was analyzed. Typing of these DNAs is illustrated in Fig. 5 and 6 (lanes 1 to 9, respectively). Amplification of the central region of the CS gene encoding the repeats readily identified size polymorphism (Fig. 5C). Six distinct alleles (arbitrarily named a to f in Table 2) could be identified by the single criterion of size polymorphism, reflecting variable numbers of repeats. Three isolates (DNAs 2, 5, and 8) yielded two bands, and one isolate (DNA 9) yielded three fragments. The intensities of the various bands differed, e.g., in DNAs 5 and 8, there was a major band and a faint one, while in DNA 9 the three bands differed in intensity. The sizes of the respective fragments are indicated in Table 2. Amplification of TRAP, shown in Fig. 5A, also detected size polymorphism. Five distinct sizes (named a to e in Table 2) were observed. DNA 2 generated three bands, while DNA 9 yielded two bands. As indicated above, the TRAP gene presents convenient polymorphic restriction sites. The polymorphism of the *Hin*fI and *Bgl*II sites has been reported previously (29) and is depicted in Fig. 1. In the region amplified in the present study, *Hin*fI and *Bgl*II RFLPs were indeed observed (data not shown), but were less informative than *Taq*I restriction. As shown in Fig. 5B, the *Taq*I RFLP indicated that each sample had a distinct profile and that DNAs 1, 5, 7, and 8, all of which generated a 740-bp product, contained distinct TRAP alleles. Table 2 summarizes the typing data obtained for CS and TRAP, which showed that all DNAs were different from each other.

Typing with MSA-1 A–MSA-1 B and MSA-2 1–MSA-2 4 confirmed and expanded this conclusion. In contrast to the relatively simple profiles observed for the previous markers, a large number of bands were visualized, with a specific pattern noted for each DNA (Fig. 6). Some samples generated up to
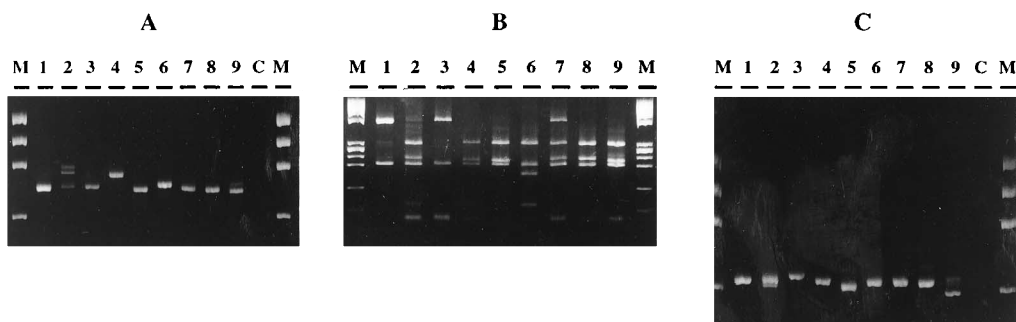

FIG. 5. Molecular typing of nine isolates from patients with acute malaria in Dielmo village. (A) Analysis of the amplification products generated from the nine DNAs with TRAP 3–TRAP 4. (B) RFLP analysis of the TRAP PCR products with *Taq*I. (C) Analysis of the amplification products generated from the nine DNAs with CS 3–CS 4. Lanes 1 to 9 were loaded with DNAs 1 to 9, respectively, in each gel. Lane M, the molecular weight markers (*Hae*III-digested φX174 replicative-form DNA).
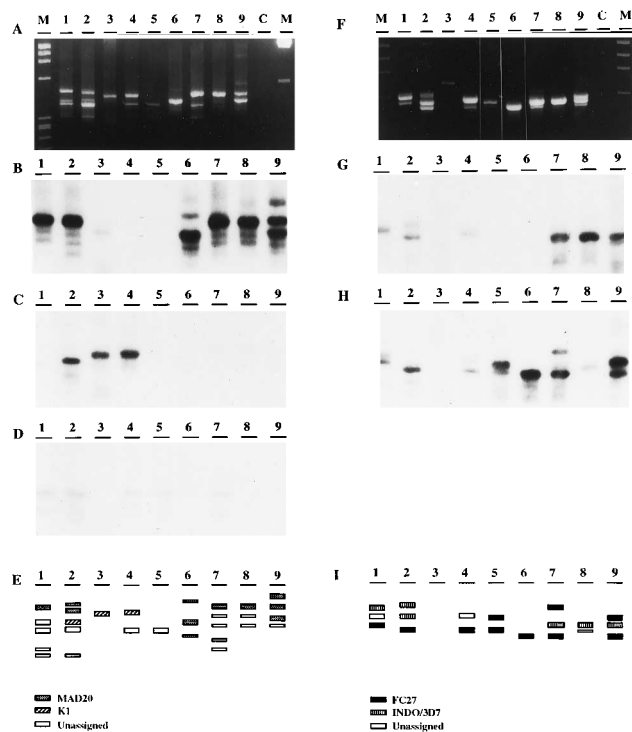
FIG. 6. PCR analysis of the MSA-1 and MSA-2 (1+4) markers in the nine isolates from patients in the Dielmo village. (A to E) The amplification products generated with primers (A and B) were analyzed. (A) Ethidium bromide staining. Lanes 1 to 9, DNAs 1 to 9, respectively; lane C, control PCR with no added DNA; lane M, molecular weight markers (HaeIII-digested φX174 replicative-form DNA on the left and HindIII-digested bacteriophage lambda DNA on the right). (B, C, and D) Autoradiograms after high-stringency washing (0.1× SSC at 65°C) of the filters prepared from the gel shown in panel A hybridized with the radiolabelled family-specific probes: MAD20 type (B) and K1 type and RO33 type (C). (E) Schematic interpretation of the results. (F to I) Analysis of the MSA-2 products generated with primers (1 and 4). (F) Ethidium bromide staining (as in panel A); (G and H) autoradiograms after high-stringency washing of the filters hybridized with the radiolabelled family-specific probes of the INDO/3D7 type (G) and the FC27 type (H). (I) Interpretation of the results. Unassigned indicates a fragment hybridizing to one or the other probe (and sometimes to both) under nonstringent conditions (2× or 0.5× SSC at 65°C) that no longer hybridized at a higher stringency.

TABLE 2. Typing data for CS and TRAP

DNA	CS		TRAP		
	Size (bp)	Allele	Size (bp)	Allele	RFLP pattern
1	600	a	740	a	1
2	600 + 560	a + b	760 + 840 + 860	b + c + d	2
3	610	c	760	b	3
4	600	a	830	e	4
5	620 + 560	d + b	740	a	5
6	600	a	760	b	6
7	600	a	740	a	7
8	630 + 600	e + a	740	a	8
9	600 + 560 + 540	a + b + f	740 + 760	a + b	9

DISCUSSION

Parasite typing is an essential component in an investigation of host-parasite relationships. Because of the small volume of blood needed and the possibility of using material collected directly from the patient without requiring in vitro manipulation, PCR-based typing presents attractive advantages. This requires, however, that both the quantitative and qualitative parameters be evaluated. In the present study we investigated some of the parameters influencing the amplification of highly polymorphic loci to delineate the framework in which data are to be interpreted.

The PCRs described here are specific for *P. falciparum* parasites, indicating that they are suitable for analyzing blood samples containing more than one species that causes malaria. This is important, because in many areas where malaria is endemic, several species that cause malaria are present. This is the case in Dielmo (39). PCR was semiquantitative, because for all markers studied there was a dilution range in which the intensity of the ethidium bromide staining was approximately proportional to the quantity of the added genomic DNA. The sensitivity obtained in the various reactions compared favorably with those reported by other groups (28, 31, 40). Importantly, all reference alleles were amplified with the same efficiency. This indicates that amplification efficiency does not strongly depend on the primary sequence or on the length of the region amplified. If this is valid for all allelic forms, the reactions that we used allow for a study of allelic prevalence in the field. This conclusion is balanced, however, by one major limitation of the method, namely, that the reaction yields were strongly influenced by the relative ratio of alleles in allelic mixtures and did not reflect the actual composition at certain allele ratios. Sensitivity depended on the primer combinations used within one locus and differed from one locus to the other. This was not unexpected, but it creates problems in comparing the markers, because the diversity of the various loci was not investigated with the same resolution. Hence, the results should be interpreted with these limitations in mind.

Typing of nine isolates from patients with acute malaria from the Dielmo village (39) clearly illustrated the genetic diversity of malaria parasites in the field. The CS gene presented six distinct size polymorphisms. The amplified portion of the TRAP gene also showed five distinct sizes, and each sample had a unique RFLP profile. TRAP RFLP based on *Taq*I on its own discriminated the nine isolates. This is interesting because limited *Taq*I restriction site polymorphism has been described in the region investigated in the present study (29) and indicates that the polymorphism of field isolates may be significantly larger than that described for the various laboratory strains. Similarly, a significant diversity was observed

five distinct bands. Apart from the MSA-2 product obtained from DNA 3, which failed to hybridize, each of the bands visualized by ethidium bromide staining hybridized under moderately stringent conditions (0.5× SSC at 65°C) with one or the other reference probes and sometimes with two of them (data not shown). This indicated that, even under nonstringent conditions, the hybridization profiles were specific. We concluded, therefore, that they were bona fide MSA-1 or MSA-2 alleles. The MSA-2 fragment obtained in DNA 3 needs to be sequenced in order to identify it as an MSA-2 allele. Under stringent conditions (0.1× SSC at 65°C), only those few alleles that formed highly stable hybrids with the probe remained detected (Fig. 6B to D and Fig. 6G and H, for MSA-1 and MSA-2, respectively). The signals were unambiguous. The schematic typing for each locus is indicated in Fig. 6E and I, respectively. The "unassigned" alleles were those that hybridized to two distinct probes under moderately stringent conditions.

Attempts to look for distinct MSA-2 alleles within these allelic families by using *Ava*I or *Hph*I RFLP (22) were unsuccessful. The complexity of the allelic mixtures generated multiple band profiles that did not permit allele assignments.

for MSA-1 and MSA-2, which gave multiple band patterns specific for each sample. With the exception of one MSA-2 fragment, every other MSA-1 or MSA-2 PCR fragment hybridized to one of the specific probes. This confirmed that the multiple bands visualized were indeed amplified MSA-1 or MSA-2 alleles. We did not detect any RO33-type MSA-1 allele. This contrasts with the report of a high prevalence of the RO33 allele in Senegal (15). Because we demonstrated here that the three major alleles of MSA-1 are amplified with similar yields, the discrepancy is not due to a technical limitation, but reflects intrinsic differences in parasites from distinct geographical origins. Alternatively, it could possibly be explained by the fact that the ratio of the RO33 allele is always unfavorable. A significant proportion of fragments (3 of 19 for MSA-2 and 15 of 29 for MSA-1) did not hybridize under stringent conditions, and these most probably constitute hybrid genes (19, 24). An alternative useful strategy for studying these genes is to perform a nested PCR with allele-specific primers (25).

An unexpected finding in the analysis of the samples from Dielmo was the frequency of multiple band patterns that were observed in two samples for TRAP, four samples for CS, and seven samples for MSA-1 and MSA-2. This indicated that none of the isolates that we studied contained a single parasite type. Up to three distinct MSA-2, TRAP, or CS bands and up to five distinct MSA-1 fragments were detected simultaneously and in many samples more than one major allelic family was detected. The presence of multiple fragments complicated the assignment of specific alleles to specific bands. Interestingly, the multiple band patterns were all different and hence provide an easy and rapid means of discriminating between isolates.

The observation of such a high proportion of infections with multiple clones is unprecedented. Most isolates analyzed so far by PCR showed that the prevalence of mixed infections is low. We have observed only 12.5% mixed parasite populations in samples collected from other regions of Senegal, such as Pikine, Dakar, and Bandia (22, 23), consistent with the results reported by other groups for isolates collected in other countries where malaria transmission is low (13, 34). The large proportion of mixed infections in samples collected from symptomatic patients in Dielmo was confirmed in a longitudinal analysis of clinical infections in children (4a) and asymptomatic children, who harbored parasites without presenting clinical symptoms (25). This suggests that the complexity of the infections may be related to the intensity of transmission.

ACKNOWLEDGMENTS

REFERENCES

1. **Arnot, D. E., C. Roper, and R. A. L. Bayoumi.** 1993. Digital codes from hypervariable tandemly repeated DNA sequences in the *Plasmodium falciparum* circumsporozoite gene can genetically barcode isolates. Mol. Biochem. Parasitol. **61:**15–24.
2. **Babiker, H. A., A. M. Creasey, B. Fenton, R. A. L. Bayoumi, D. E. Arnot, and D. Walliker.** 1991. Genetic diversity of *Plasmodium falciparum* in a village in eastern Sudan. 1. Diversity of enzymes, 2D-PAGE proteins and antigens. Trans. R. Soc. Trop. Med. Hyg. **85:**572–577.
3. **Barker, R. H., Jr., T. Banchongaksorn, J. M. Courval, W. Suwonkerd, K. Rimwungtrgoon, and D. F. Wirth.** 1992. A simple method to detect *Plasmodium falciparum* directly from blood samples using the polymerase chain reaction. Am. J. Trop. Med. Hyg. **46:**416–426.
4. **Carter, R., and I. A. McGregor.** 1973. Enzyme variation in *Plasmodium falciparum* in the Gambia. Trans. R. Soc. Trop. Med. Hyg. **67:**830–837.
4a. **Contamin, H., et al.** Submitted for publication.
5. **Conway, D. J., B. M. Greenwood, and J. S. McBride.** 1991. The epidemiology of multiple clone *Plasmodium falciparum* infections in Gambian patients. Parasitology **103:**1–6.
6. **Conway, D. J., B. M. Greenwood, and J. S. McBride.** 1992. Longitudinal study of *Plasmodium falciparum* polymorphic antigens in a malaria-endemic population. Infect. Immun. **60:**1122–1127.
7. **Conway, D. J., and J. S. McBride.** 1991. Population genetics of *Plasmodium falciparum* within a malaria hyperendemic area. Parasitology **103:**7–16.
8. **Creasey, A., B. Fenton, A. Walker, S. Thaithong, S. Oliveira, S. Mutambu, and D. Walliker.** 1990. Genetic diversity of *Plasmodium falciparum* shows geographical variation. Am. J. Trop. Med. Hyg. **42:**403–413.
9. **Dame, J. B., J. L. Williams, T. F. McCutchan, J. L. Weber, R. A. Wirtz, W. T. Hockmeyer, W. L. Maloy, J. D. Haynes, J. Schneider, I. Roberts, D. Sanders, G. S. Reddy, E. P. Diggs, and L. H. Miller.** 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum*. Science **225:**593–599.
10. **Fandeur, T., S. Bonnefoy, and O. Mercereau-Puijalon.** 1991. In vivo and in vitro derived Palo Alto lines of *Plasmodium falciparum* are genetically unrelated. Mol. Biochem. Parasitol. **47:**167–178.
11. **Fenton, B., J. T. Clark, C. M. A. Khan, J. V. Robinson, D. Walliker, R. Ridley, J. G. Scaife, and J. McBride.** 1991. Structural and antigenic polymorphism of the 35- to 48-kilodalton merozoite surface antigen (MSA-2) of the malaria parasite *Plasmodium falciparum*. Mol. Cell. Biol. **11:**963–971.
12. **Galinski, M. R., C. Coredor Medina, P. Ingravallo, and J. W. Barnwell.** 1992. A reticulocyte-binding protein complex of *Plasmodium falciparum*. Cell **69:** 1213–1226.
13. **Kahane, B., L. Sibilli, A. Scherf, G. Jaureguiberry, G. Langsley, L. S. Ozaki, M. Guillotte, B. Müller-Hill, L. Pereira da Silva, and O. Mercereau-Puijalon.** 1987. The polymorphic 11.1 locus of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **26:**77–86.
14. **Kemp, D. J., A. F. Cowman, and D. Walliker.** 1990. Genetic diversity in *Plasmodium falciparum*. Adv. Parasitol. **29:**75–147.
15. **Kimura, E., D. Mattei, S. mana di Santi, and A. Scherf.** 1990. Genetic diversity in the major merozoite surface antigen of *Plasmodium falciparum*: high prevalence of a third polymorphic form detected in strains derived from malaria patients. Gene **91:**57–62.
16. **Kyes, S., A. G. Graig, K. Marsh, and C. I. Newbold.** 1993. *Plasmodium falciparum*: a method for the amplification of S antigens and its application to laboratory and field samples. Exp. Parasitol. **77:**473–483.
17. **Lockyer, M. J., K. Marsh, and C. I. Newbold.** 1989. Wild isolates of *Plasmodium falciparum* show extensive polymorphism in T cell epitopes of the circumsporozoite protein. Mol. Biochem. Parasitol. **37:**275–280.
18. **Marshall, V. M., R. L. Anthony, M. J. Bangs, Purnomo, R. F. Anders, and R. L. Coppel.** 1994. Allelic variants of the *Plasmodium falciparum* merozoite surface antigen 2 (MSA-2) in a geographically restricted area of Irain Jaya. Mol. Biochem. Parasitol. **63:**13–21.
19. **Marshall, V. M., R. L. Coppel, R. K. Martin, A. M. J. Oduola, R. F. Anders, and D. J. Kemp.** 1991. A *Plasmodium falciparum* MSA-2 gene apparently generated by intragenic recombination between the two allelic families. Mol. Biochem. Parasitol. **45:**349–352.
20. **McBride, J. S., P. D. Welsby, and D. Walliker.** 1984. Serotyping of *Plasmodium falciparum* from acute human infections using monoclonal antibodies. Trans. R. Soc. Trop. Med. Hyg. **78:**32–34.
21. **McCutchan, T. F., A. A. Laf, V. do Rosario, and A. P. Waters.** 1992. Two types of sequence polymorphism in the circumsporozoite gene of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **50:**37–46.
22. **Mercereau-Puijalon, O., T. Fandeur, S. Bonnefoy, C. Jacquemot, and J. L. Sartou.** 1991. A study of the genomic diversity of *Plasmodium falciparum* in Senegal. 2. Typing by the use of the polymerase chain reaction. Acta Trop. **49:**293–304.
23. **Mercereau-Puijalon, O., and J. L. Sarthou.** Unpublished data.
24. **Miller, L. H., T. Roberts, M. Shahabuddin, and T. Mccutchan.** 1993. Analysis of sequence diversity in the *Plasmodium falciparum* merozoite surface protein-1 (MSP-1). Mol. Biochem. Parasitol. **59:**1–14.
25. **Ntoumi, F., H. Contamin, C. Rogier, S. Bonnefoy, J. F. Trape, and O. Mercereau-Puijalon.** Age-dependent carriage of multiple *Plasmodium falciparum* MSA-2 alleles in asymptomatic malaria infections. Am. J. Trop. Med. Hyg., in press.
26. **Nussenzweig, R. S., and N. Yoshida.** 1990. Variation of T cell epitopes of the *Plasmodium falciparum* CS protein: its occurrence, extent and relevance. Immunol. Lett. **25:**21–22.
27. **Prescott, N., A. W. Stowers, Q. Cheng, A. Bobogare, C. M. Rzepczyk, and A. Saul.** 1994. *Plasmodium falciparum* genetic diversity can be characterized using the polymorphic merozoite surface antigen 2 (MSA-2) gene as a single

locus marker. Mol. Biochem. Parasitol. **63:**203–212.

28. **Ranford-Cartwright, L. C., P. Balfe, R. Carter, and D. Walliker.** 1991. Genetic hybrids of *Plasmodium falciparum* identified by amplification of genomic DNA from single oocyst. Mol. Biochem. Parasitol. **49:**239–244.

29. **Robson, K. J. H., J. R. S. Hall, L. C. Davies, A. Crisanti, A. V. S. Hill, and T. E. Welllems.** 1990. Polymorphism of the TRAP gene of *Plasmodium falciparum*. Proc. R. Soc. London **242:**205–216.

30. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.

31. **Schriefer, M. E., J. B. Sacci, Jr., R. A. Wirtz, and A. F. Azad.** 1991. Detection of polymerase chain reaction-amplified malaria DNA in infected blood and individual mosquitoes. Exp. Parasitol. **73:**311–316.

32. **Sethabutr, O., A. E. Brown, S. Panyim, K. C. Kain, K. Webster, and P. Echeverria.** 1992. Detection of *Plasmodium falciparum* by polymerase chain reaction in a field study. J. Infect. Dis. **166:**145–148.

33. **Smythe, J. A., R. L. Coppel, K. P. Day, R. K. Martin, A. M. J. Oduola, D. J. Kemp, and R. F. Anders.** 1991. Structural diversity in the *Plasmodium falciparum* merozoite surface antigen 2. Proc. Natl. Acad. Sci. USA **88:**1751–1755.

34. **Snewin, V. A., M. Herrera, G. Sanchez, A. Scherf, G. Langsley, and S. Herrera.** 1991. Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. Mol. Biochem. Parasitol. **49:**265–276.

35. **Snounou, G., S. Viriyakosol, W. Jarra, S. Thaithong, and K. N. Brown.** 1993. Identification of the four human malaria parasite species in field samples by the polymerase chain reaction and detection of a high prevalence of mixed infections. Mol. Biochem. Parasitol. **58:**283–292.

36. **Snounou, G., S. Viriyakosol, X. P. Zhu, W. Jarra, L. Pinheiro, V. E. Rosario, S. Thaithong, and K. N. Brown.** 1993. High sensitivity of detection of human malaria parasites by use of nested polymerase chain reaction. Mol. Biochem. Parasitol. **61:**315–320.

37. **Tanabe, K., M. Mackay, M. Goman, and J. G. Scaife.** 1987. Allelic dimorphism in a surface antigen gene of the malaria parasite *Plasmodium falciparum*. J. Mol. Biol. **195:**273–287.

38. **Tirasophon, W., A. Tassanakajon, V. Boonsaeng, S. Panyim, and P. Wilairat.** 1993. Sensitive detection of *Plasmodium falciparum* in blood and mosquito by DNA amplification. Parassitologia **35:**117–119.

39. **Trape, J. F., C. Rogier, L. Konate, N. Diagne, H. Bouganali, B. Canque, F. Legros, A. Badji, G. Ndiaye, P. Ndiaye, K. Brahimi, O. Faye, P. Druilhe, and L. Pereira da Silva.** 1994. The Dielmo project. A longitudinal study of natural malaria infection in a community living in a holoendemic area of Senegal. Am. J. Trop. Med. Hyg. **51:**123–137.

40. **Wataya, Y., M. Arai, F. Kubochi, C. Mizukoshi, T. Kakutani, N. Ohta, and A. Ishii.** 1993. DNA diagnosis of falciparum malaria using a double PCR technique: a field trial in the Solomon Islands. Mol. Biochem. Parasitol. **58:**165–168.

41. **Wooden, J., E. E. Gould, A. T. Paull, and C. H. Sibley.** 1992. *Plasmodium falciparum*: a simple polymerase chain reaction method for differentiating strains. Exp. Parasitol. **75:**207–212.