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DIFFERENT GENETIC CHARACTERISTICS OF *PLASMODIUM FALCIPARUM* ISOLATES COLLECTED DURING SUCCESSIVE CLINICAL MALARIA EPISODES IN SENEGALESE CHILDREN

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Abstract. A narrow epidemiologic survey was conducted during a four-month period of intense malaria transmission in Dielmo, a holoendemic Senegalese village. Longitudinal clinical and parasitologic follow-up indicate that clinical malaria episodes always occurred after an abrupt increase in parasite densities. Polymerase chain reaction analysis of *Plasmodium falciparum* parasites was carried out in blood samples collected longitudinally from 10 children who had experienced several clinical episodes during this period. Our data show that the genetic diversity of the parasites circulating in this village is very large. The successive clinical episodes experienced by each child were caused by genetically distinct parasite populations that were recently inoculated and multiplied in an apparently unrestricted manner. Importantly, the genetic characteristics of the parasite populations detected during phases of asymptomatic carriage differed from those causing a clinical episode, suggesting that the various factors that control of parasite growth in these children are strain-specific.

In naive individuals, infection by malaria parasites almost invariably results in a clinical episode. In humans living in regions where malaria transmission is high, clinical malaria primarily affects young children and the presence of blood-stage parasites is not synonymous with disease. A significant proportion of young children, most older children, and many adults carry parasites without presenting clinical symptoms.[1] It is generally admitted that this reflects the efficiency of the progressively acquired malaria-specific immunity, which prevents clinical manifestations and reduces parasite burden. To date, little is known about how this protective immunity is elicited, how it operates, and how parasitization results in disease.[2,3] Experimental infections in humans with defined parasite strains have shown that an efficient strain-specific immunity was acquired after infection with one strain, allowing the individual to resist infection by that strain but providing little protection against a heterologous one.[4,5] Several infections with the same strain were, however, required to elicit a sterile immunity to that strain.[5] These findings showed that both poor immunogenicity and strain diversity may represent serious obstacles to the development of protective immunity. The relevance of these observations to the situation faced by humans living in malaria-endemic regions is still obscure. While there is now ample evidence that malaria parasite species are polymorphic for a large number of characteristics,[6] the extent of genetic diversity of local parasite populations to which people are actually exposed is essentially unknown, and virtually nothing is known about the circulation of strains in a restricted geographic area.[7,8] Therefore, the question of whether parasite diversity plays a major role in the occurrence of clinical episodes in endemic regions, a key element in our understanding of host/parasite relationships, is still unanswered.

To address this issue, we have carried out an analysis of the genetic characteristics of *Plasmodium falciparum* parasites collected longitudinally from children who experienced several successive clinical episodes. In Dielmo, a Senegalese village where malaria is holoendemic, a close follow-up of the entire population of the village was carried out during the 1990 rainy season.[9] As a result, longitudinal records of parasite densities of *P. falciparum, P. malariae,* and *P. ovale* and of clinical events are available for each inhabitant of the village during this four-month period of high transmission.[9] The mean inoculation rate was approximately one *P. falciparum* infective bite every second night and most children 1–6 years of age experienced more than one clinical malaria episode. During this survey, capillary blood samples were collected at two-week intervals as well as during clinical episodes, allowing a longitudinal analysis of the characteristics of the parasites infecting the inhabitants of this village over a four-month period of intense malaria transmission.

Plasmodium falciparum parasites circulating in human peripheral blood are haploid ring stages. We have carried out a typing procedure based on the polymerase chain reaction (PCR) that uses DNA from circulating parasites to analyze several single-copy genetic loci. The large allelic diversity reported in regions such as block 2 of the merozoite surface antigen-1 (MSA-1) gene or the central repetitive domain of the MSA-2 gene, differing both in nucleotide sequence and in number of repetitive sequences, or in the thrombospondin related anonymous protein (TRAP) gene, presenting restriction site polymorphism, renders these markers particularly convenient for typing purposes.[10] Distinct parasite populations can readily be identified using one or a combination of these characters.[10,11] Such a typing approach was used in the work reported here to analyze the characteristics of *P. falciparum* parasites collected from 10 children who had experienced two or more clinical malaria episodes during the four-month period of intense follow-up in Dielmo.

MATERIALS AND METHODS

Study site. The Dielmo village is located in the Fatick region, 280 km southeast of Dakar, Senegal. After reaching

an agreement with the leaders and the population of the village, as well as with the national authorities, an epidemiologic study was undertaken in June 1990. A detailed description of Dielmo and of the epidemiologic study conducted in this village has been reported elsewhere.[9] Due to the presence of a stream, malaria transmission is perennial. From June 1 to September 30, 1990, when the samples analyzed were collected, the inoculation rate was approximately one infective *P. falciparum* bite every second night.

Longitudinal clinical and parasitologic follow-up. The clinical, parasitologic information reported here for 10 children was collected during the close follow-up of the entire population of the village (247 inhabitants) carried out from June 1 to September 30, 1990. Briefly, each person was visited at home three times a week to record temperature (rectal temperature for the children less than seven years of age) and to administer a clinical questionnaire concerning the past 48 hr. In addition, each compound was visited daily to rapidly detect new cases of fever. Twice a week, a thick blood smear was made from each person in the village. In all patients with fever, additional blood smears were taken. The thick blood smears were stained with Giemsa and at least 200 oil-immersion fields were examined by microscopy. Parasites were quantified as described by Trape and others.[9, 12] Since parasite prevalence in the village was very high, a cutoff in parasite density was used to define malaria episodes and decide upon treatment. For children, a clinical malaria episode was defined as fever (rectal temperature $\geq$ 38.5°C) associated with a parasite:leukocyte ratio $\geq$ 2, i.e., $\geq$ 16,000 parasites/μl.[12] Treatment consisted in administration of Quinimax® (Sanofi-Labaz, Paris, France) at a dose of 25 mg/kg/day in three daily doses over three days. As part of the protocol, the Dielmo villagers were asked not to use chemoprophylaxis or automedication without informing the team. Use of antimalarials by the population was indeed restricted to our prescriptions, as indicated by questionnaire and monthly urine tests.[9]

Collection of blood samples. Blood samples were collected twice a month using capillary pipettes and venous blood samples were collected during malaria attacks. The samples were centrifuged, the buffy coat was discarded, and the red blood cell pellet was frozen in liquid nitrogen in the village. After transportation, the tubes were stored at −80°C. The protocol was approved by the Ministere de la Cooperation et du Developpement and the Ministere de la Sante Publique of Senegal. Informed consent was obtained individually from the participants or their parents.

Extraction of DNA. Using standard procedures,[10] DNA was extracted. Briefly, approximately 100 μl of red blood cells were lysed by three freeze-thaw cycles. Free parasites were washed three times with distilled water and resuspended in five 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/ml of proteinase K. After 1 hr of incubation at 37°C, the DNA was extracted twice with one volume of phenol/chloroform/isoamyl alcohol (25/24/1), and precipitated with ethanol in the presence of 0.3 M sodium acetate (pH 5.5). The DNA was resuspended in 50 μl of water.

Amplification by PCR. The PCR procedures, permitting analysis of polymorphic regions of the MSA-1, MSA-2, and TRAP loci, are detailed elsewhere.[10] Briefly, amplification was done using 1–5 μl of DNA in a final volume of 50 μl in the presence of 200 μM of each dNTP, 1 μM of each primer, and 2.5 U of *Taq* I DNA polymerase (Promega France, Charbonnieres, France) in the buffer supplied by Promega (12 mM $MgCl_2$). The reactions were performed in a Hybaid thermocycler (Cera-Labo, Aubervilliers, France). The standard reactions parameters were 15 sec at 94°C, 2 min at 55°C, 2 min at 72°C (one cycle), 2 sec at 94°C, 2 min at 55°C, 2 min at 72°C (35 cycles), and 2 sec at 94°C, 2 min at 55°C, and 10 min at 72°C (one cycle).

The primers used for MSA-1 were MSA-1 A: 5′AAG CTT TAG AAG ATG CAG TAT TGA C3′ and MSA-1 B: 5′ATT CAT TAA TTT CTT CAT ATC CAT C3′. For the MSA-2 gene the following primers were used: MSA-2 1: 5′ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA3′ and MSA-2 2: 5′AAC GAA TTC ATA AAC AAT GCT TAT AAT ATG AGT3′; MSA-2 3: 5′GAT GAA TTC TAG AAC CAT GCA TAT GTC CAT GTT3′ and MSA-2 4: 5′ATA TGG CAA AAG ATA AAA CAA GTG TTG CTG3′. For the amplification of the TRAP gene, the following primers were used: TRAP 3: 5′ATG TAA CTT GTA TGC TGA TTC TGC ATG G3′ and TRAP 4: 5′TAT CTT CAC TAT TAG GTA CGT GCC TAT TTC C3′.[10]

Analysis of PCR products and allele assignment. The PCR products were analyzed for size polymorphism on agarose gels (Seakem GTG-Tebu France, Le Perray en Yvelines, France) as described.[10] The TRAP alleles were identified by restriction fragment length polymorphism (RFLP) after digestion with *Taq* I restriction endonuclease.[10] The MSA-1 and MSA-2 alleles were assigned to allelic families by hybridization using family-specific probes, as described.[10] Probes were prepared by nick translation (nick translation kit; Boehringer Mannheim, Meylan, France) using specific fragments amplified from reference clones or isolates, whose sequence has been determined or from the recombinant plasmids obtained after cloning the various MSA-1 and MSA-2 reference probes (TA cloning kit; In Vitrogen, Oxon, United Kingdom). A PCR product was assigned to a specific allelic family if it hybridized with the corresponding probe under stringent conditions (0.1× SSC [15 mM NaCl, 15 mM sodium citrate, pH 7.0] at 65°C). The fragments that hybridized to one or more probe at moderate stringency (0.5× SSC at 65°C), but no longer at high stringency (0.1× SSC at 65°C) were considered hybrids. Fragments that hybridized to distinct probes under conditions of low stringency (6× SSC at 65°C), produced faint hybridization signals at higher stringency (2× SSC at 65°C), and did not hybridize under more stringent conditions were grouped as unassigned alleles. They most probably consisted of complex mosaic hybrid genes.[10]

RESULTS

A total of 132 *P. falciparum* clinical malaria episodes were observed in the 247 inhabitants of Dielmo. Most (92.6%) occurred in children less than 10 years of age. Babies and children less than four years old experienced a mean of two clinical attacks, while older children (5–9 years of age) had a mean of one clinical attack.[9] In the birth to 6-year-old group, 34 (61%) of 56 children had more than one

clinical attack. None of the children more than seven years of age experienced more than one attack.[13]

The longitudinal parasitologic follow-up conducted during this period showed that malaria attacks were always associated with an abrupt increase in parasite density. In most cases, drug treatment resulted in rapid resolution of symptoms and clearance of asexual blood stages in the first few days. This is illustrated in Figures 1–6, in which the longitudinal records of parasite densities of the children studied are presented.

In this work, we have investigated some genetic characteristics of *P falciparum* parasites collected longitudinally from children who had experienced two or more clinical episodes during the study period. Ten representative children, who presented between clinical episodes blood smears that were either 1) constantly negative or 2) constantly positive, but with low parasite densities (trophozoites and/or gametocytes), were chosen. A total of 89 DNA preparations were made, including 28 from *P. falciparum* clinical malaria episodes. The PCR typing was carried out for three distinct genetic loci located on distinct chromosomes.[10] For MSA-1 (also called MSP-1), the highly polymorphic block 2 was amplified. For MSA-2 (also called MSP-2), two independent reactions were done, amplifying the entire coding region (primers 1 + 4) or the central polymorphic domain (primers 2 + 3). The TRAP amplification concerned the central region of the gene. All PCR primers used here were highly specific for *P. falciparum* parasites, as tests with other human malaria species did not yield any PCR product.[10] Analysis of the amplified fragments was done by investigating size polymorphism in all cases, and RFLP was investigated for the TRAP reaction. Both MSA-1 and MSA-2 fragments were assigned to specific allelic families (MAD 20, K1, and RO33 for MSA-1 and 3D7 and FC27 for MSA-2) by hybridization using family-specific probes.[10]

Analysis of the parasites collected during successive malaria episodes. We first analyzed the clinical episodes of children presenting constantly negative smears between the attacks. A typical example is illustrated in Figure 1, showing parasite typing from a 1.5-year-old girl, code 02/04, who experienced two clinical malaria episodes (arbitrarily designated A and B in Figure 1), with high parasite densities, both of which were successfully drug-treated. Apart from the smears collected during the attacks or for the following 2–3 days, all thick blood smears collected from this child were negative. Eight successive blood samples (numbered 1–8) were available. The DNAs 1–3 and 6–7 were collected as part of the systematic survey, and the corresponding thick blood smears were negative. The PCR from these samples was also negative for all markers investigated (Figure 1, lanes 1–3 and 6-7, respectively). Figure 1 shows that DNAs 4 and 5, collected two days apart during episode A, presented the same amplification patterns for MSA-1 and MSA-2, with two bands for each marker. The pattern obtained for DNA 8, collected during episode B, however, produced a single band of a different size for both reactions. Assignment to specific allelic families was done by hybridization using family-specific probes,[10] as summarized in Table 1. The MSA-1 fragments generated from DNAs 4 and 5 hybridized with the K1 family-specific probe, while the fragment obtained from DNA 8 hybridized with the MAD20 family-

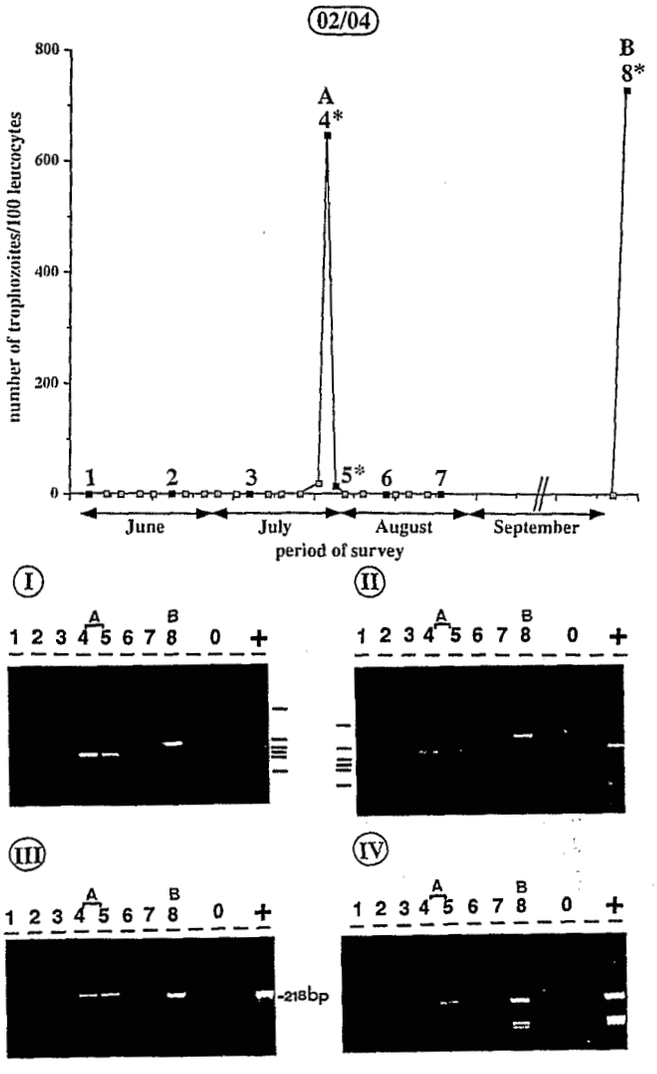


FIGURE 1. Course of *Plasmodium falciparum* parasitemia **(top)** and typing of the parasites **(bottom)** collected from child 02/04, a 1.5-year-old girl, who experienced two clinical attacks, denoted A and B. Thick blood smears were collected twice a week. Positive thick blood smears contained exclusively *P. falciparum* trophozoites. Parasite densities are expressed as the number of *P. falciparum* trophozoites for 100 leukocytes (i.e., 1/50–1/100 μl of blood). DNAs were prepared from blood samples collected on June 4 (1), June 22 (2), July 9 (3), July 26 (4, episode A), July 28 (5), August 10 (6), August 24 (7), and September 29 (8, episode B). Clinical episodes were drug-treated, as described in the Materials and Methods. Numbers with an asterisk indicate that the corresponding thick blood smear was positive, while for the others, thick blood smears were negative. **Bottom,** analysis of the polymerase chain reaction (PCR) products by electrophoresis on agarose gels and staining with ethidium bromide. I, amplification of merozoite surface antigen-1 (MSA-1) using primers A + B. II, amplification of MSA-2 using primers 2 + 3; III and IV, typing for thrombospondin related anonymous protein (TRAP), undigested, and *Taq* I-restricted PCR products, respectively. The numbers above each lane refer to the number of the DNA sample analyzed. 0 = no DNA added in the reaction; + = positive control (DNA from clone 89F5).[18] The positions of the molecular weight markers (Boehringer Mannheim marker VI) are indicated schematically for the 1,033-, 653-, 517-, 453-, 394-, and 298-basepair (bp) markers in I and II. The size of the TRAP fragment is indicated in III.

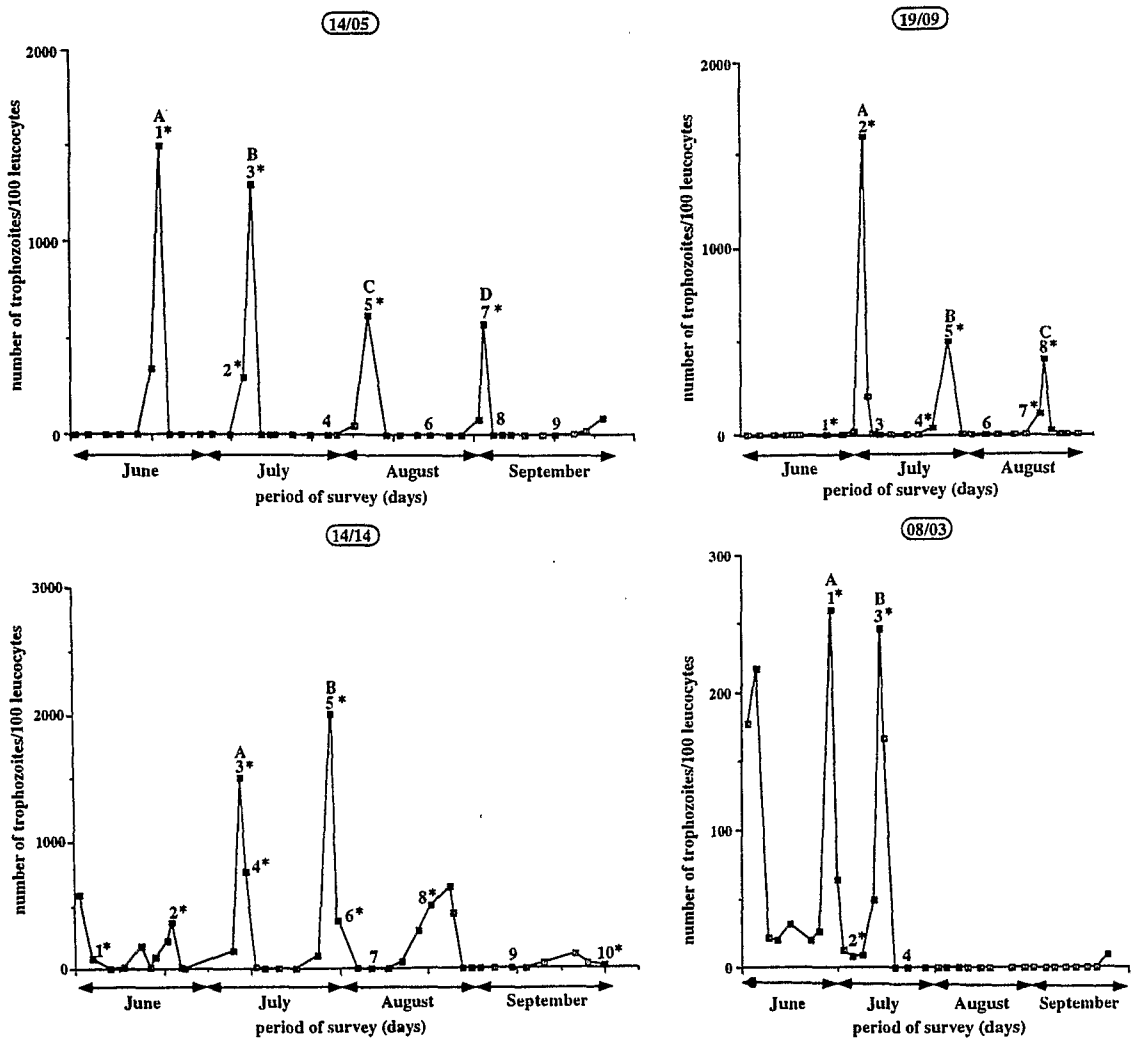
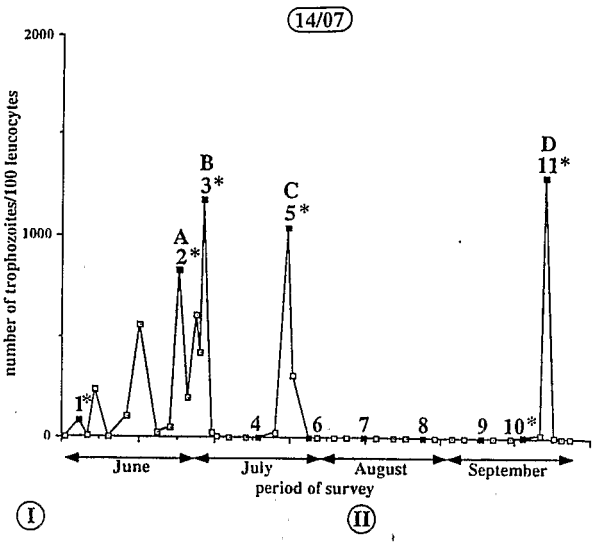


FIGURE 2. Course of *Plasmodium falciparum* parasitemia in four children. Each clinical episode was drug-treated as described in the Materials and Methods. The symbols used are as in Figure 1. **14/05,** a six-year-old boy, experienced four clinical episodes. The child carried exclusively *P. falciparum* trophozoites. Blood samples were collected on June 20 (1, episode A), July 9 (2), July 11 (3, episode B), July 28 (4), August 6 (5, episode C), August 20 (6), September 1 (7, episode D), September 3 (8), and September 17 (9). **19/09,** a three-year-old boy, experienced three clinical episodes. Blood samples were collected on June 23 (1), July 3 (2, episode A), July 7 (3), July 21 (4), July 25 (5, episode B), August 4 (6), August 18 (7), and August 19 (8, episode C). The child presented occasionally, in addition to the *P. falciparum* asexual blood stages indicated by an asterisk, low densities of *P. falciparum* gametocytes (samples 1, 3, 4, and 5) and/or *P. malariae* (samples 1 and 2) parasites during the period under study. **14/14,** a four-year-old boy, experienced four clinical episodes. Blood samples were collected on June 4 (1), June 22 (2, episode A), July 8 (3, episode B), July 9 (4), July 28 (5, episode C), July 30 (6), August 6 (7), August 20 (8, episode D), September 3 (9), and September 21 (10). The child presented, in addition to the *P. falciparum* trophozoites indicated, low densities of *P. malariae* (samples 1, 2, and 3) and *P. ovale* (sample 2) parasites. **08/03,** a five-year-old boy, experienced two clinical episodes. Blood samples were collected on June 30 (1), July 10 (2, episode A), July 16 (3, episode B), and July 25 (4). The child also carried low densities of *P. falciparum* gametocytes in all samples used for DNA extraction.

specific probe. Both DNAs 4 and 5 generated MSA-2 fragments belonging to the FC27 allelic family, and the single fragment amplified from DNA 8 was a 3D7-type. TRAP typing (Figure 1, bottom panel) confirmed that the parasites from both episodes were genetically distinct, since the alleles carried could be differentiated by *Taq* I RFLP.

As shown in Figure 2, three other children (14/05, 19/09, and 14/14) presented negative smears in the periods separating two attacks. Again, no PCR product was obtained from the samples with negative blood smears. Typing of parasites in the samples collected during clinical episodes is indicated in Table 1. Child 14/05, a six-year-old boy, experienced four episodes (designated A–D in Figure 2). Typing of these episodes (Table 1) indicated that episodes A and B could be easily distinguished both from each other, and from C and D by their TRAP, MSA-1, and MSA-2 markers. Parasites C and D could not be distinguished using MSA-1 nor TRAP, but MSA-2 typing showed that they were different, since DNA from episode C did not yield any band using primers 2 + 3 and no signal was obtained upon hybridization. Since this DNA generated a PCR product for the other markers used, including MSA-2 using primers 1 + 4, we concluded that the sequence of the MSA-2 allele from episode C was such that primers 2 + 3 did not form stable hybrids. In contrast, amplification using primers 2 + 3 was obtained from DNA collected in



DNA	date	troph.	MSA-1 A+B size (bp)	MSA-1 A+B allele	MSA-2 2+3 size (bp)	MSA-2 2+3 allele
1	4/06/90	78	420	MAD20	580	3D7
2-A	27/06/90	830	470+420	K1, MAD20	580	3D7
3-B	3/07/90	1180	470	K1	580	3D7
4	16/07/90	0	-	-	-	-
5-C	23/07/90	1046	(570)+520+490	K1	580 doublet	3D7, FC27
6	28/07/90	0	-	-	-	-
7	10/08/90	0	-	-	-	-
8	24/08/90	0	-	-	-	FC27
9	7/09/90	0	-	-	-	-
10	17/09/90	6	540	MAD20	-	-
11-D	22/09/90	1306	540	MAD20	650+450	FC27

FIGURE 3. Course of parasitemia and typing of the parasites from child 14/07, a two-year-old boy, who experienced four clinical episodes, which were drug-treated as described in the Materials and Methods. Treatment of episode A was incomplete due to vomiting. Blood samples were collected on the dates indicated. The course of parasitemia is indicated using the same symbols as in Figure 1. The child carried exclusively *Plasmodium falciparum* parasites during this period. Gametocytes were detected occasionally; none of the samples used for DNA extraction contained gametocytes. Results of amplification of merozoite surface antigen-1 using primers A + B and MSA-2 using primers 2 + 3 are shown in I and II, respectively. Fragment size was estimated on agarose gels, using Boehringer Mannheim molecular weight marker VI. The positions of the markers are indicated (from top to bottom: 1,033, 653, 517, 453, 394, and 298 basepairs [bp]). The numbers above each lane refer to the number of the DNA analyzed. 0 = no DNA added in the reaction: + = positive control (DNA from clone 89F5).[18] The bottom of the figure summarizes genotyping. troph. indicates parasitemia, expressed as the number of trophozoites/100 leukocytes. The assignment to allelic family was carried out by hybridization using family-specific probes under conditions of distinct stringency, as described in the Materials and Methods. The various alleles observed are indicated in the same order for size and allelic type. -indicates that no fragment was observed, neither by staining with ethidium bromide nor after hybridization.

episode D, which also contained multiple MSA-2 alleles detected by hybridization.

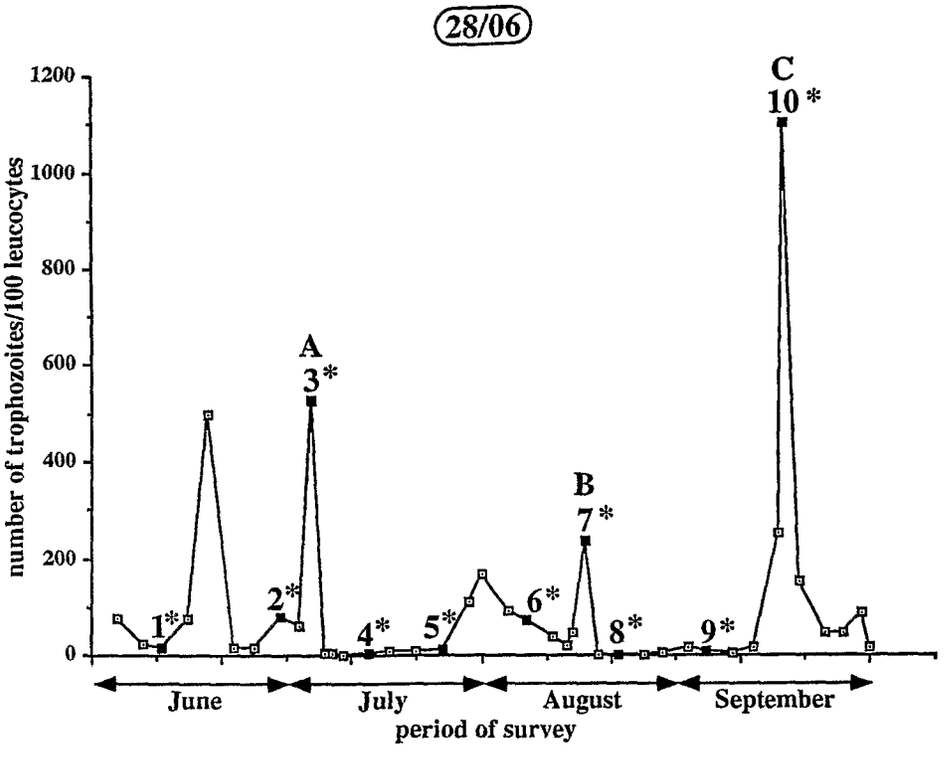
Child 19/09, a three-year-old boy, experienced three episodes (A–C in Figure 2). During each, as indicated in Table 1, the child had multiple parasite types, yielding three fragments for MSA-1, one or two fragments for MSA-2, depending on the primer combination used, and a single TRAP fragment. The MSA-1 types of episodes A and B could not be distinguished, while the alleles present during episode C differed from those detected in the previous ones. Parasites from each episode had a different TRAP pattern and a specific MSA-2 profile.

Child 14/14, a four-year-old boy, experienced four malaria episodes and had several negative blood smears in the period separating these episodes (Figure 2). Table 1 shows that each episode presented a specific TRAP allele and a unique multiple band pattern for both MSA-1 and MSA-2.

Other children presented negative blood smears shortly after treatment but could, thereafter, harbor parasites for several days or weeks without presenting symptoms before experiencing another clinical malaria episode. Typical examples are illustrated in Figures 3–6. Comparison of the genetic characteristics of the parasites collected during the successive episodes experienced by these children, indicated in Table 1, again shows that the parasites collected during successive clinical episodes in a given child were genetically different. Episodes A, B, and C in child 28/06 differed in their MSA-2 profiles, as did episodes A and B experienced by child 02/03. Episodes A and B in child 12/07 differed in their MSA-2 alleles and their TRAP profiles. For both episodes in child 22/06, parasites differed at the three loci investigated.

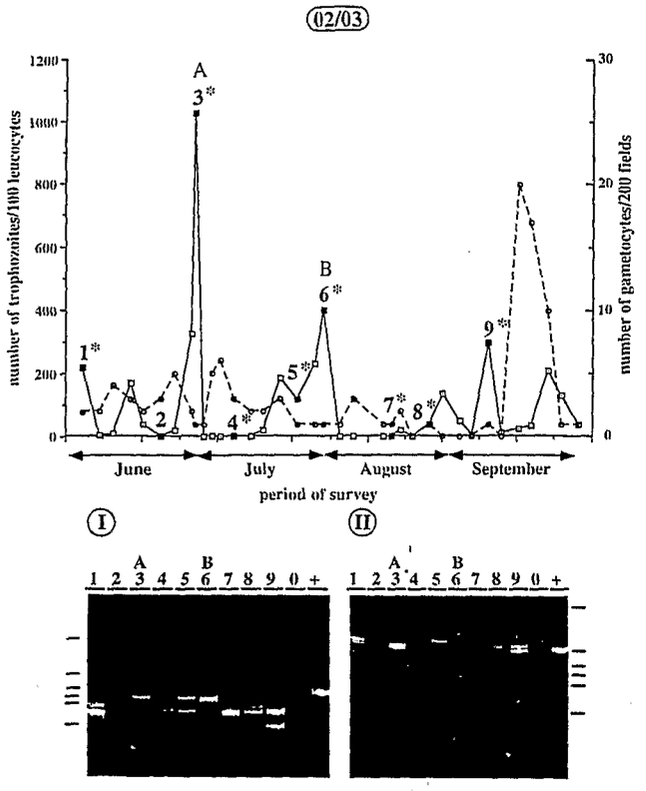
There were two cases in which drug treatment did not result in complete clearance of the sexual blood stages within the following few days. As shown in Figure 2, treatment of episode A experienced by child 08/03 was followed by a 32-fold decrease in parasite density but not by clearance, since parasites were detected in the three blood smears preceding the outgrowth provoking episode B. Typing of the parasites from episodes A (DNA 1) and B (DNA 3) shown in Table 1 indicated that for these episodes, the parasites could not be distinguished. Sample DNA 2, collected during the interval, produced the same pattern, while DNA 4, obtained after drug clearance of peak B, was negative. The results of typing are consistent with a typical recrudescence due to incomplete treatment of the first episode.

An analogous situation was observed for episodes A and B of child 14/07, a two-year-old boy. As shown in Figure 3, parasitemia in this child fluctuated during the month of June, while he remained asymptomatic. A rapid increase in parasite density resulted in a clinical episode on June 27 (episode A). Drug treatment of this episode was incompletely administered (due to vomiting) and the child did not clear the parasites. A second clinical episode (B) was recorded a few days later on July 3. Treatment of this episode was complete and the following routine thick blood smears were negative. As shown in Figure 3, I and II, the DNAs collected during episodes A and B generated the same 580-basepair (bp), 3D7-type MSA-2 allele, and both generated a 470-bp, K1-type MSA-1 allele. Parasites from episode A, however, generated an additional MSA-1 band, typed as a 420-bp



DNA	date	troph.	MSA-1 A+B size (bp)	MSA-1 A+B allele	MSA-2 1+4 size (bp)	MSA-2 1+4 allele	TRAP profile
1	11/06/90	16	480+430	K1, K1	840+820+780	3d7,FC27, FC27	a
2	29/06/90	80	430	K1	840+780	FC27, FC27	a
3-A	4/07/90	529	430	K1	840	FC27	a
4	13/07/90	2	480+410	K1, K1	840	FC27	?
5	24/07/90	11	480+410	K1, (K1,MAD20)	980+920+840	3D7, (3D7, FC27), (3D7, FC27)	?
6	6/08/90	72	480+410	K1, (K1, MAD20)	940+870+780	(3D7, FC27), (3D7, FC27), FC27	b
7-B	15/08/90	236	460+410+350	K1, (K1,MAD20), MAD20	940+820+780	3D7, FC27, FC27	c
8	20/08/90	1	-	-	-	-	-
9	3/09/90	9	460	K1	840	FC27	a
10-C	15/09/90	1100	460+410+350	K1, (K1,MAD20), MAD20	920+840	(3D7,FC27), FC27	d

FIGURE 4. Course of *Plasmodium falciparum* parasitemia and typing of the parasites from child 28/06, a six-year-old boy, who experienced three clinical episodes, which were drug-treated as indicated in the Materials and Methods. The course of parasitemia is indicated using the same symbols as in Figure 1. The child presented, in addition to the *P. falciparum* asexual blood stages indicated here, low densities of *P. falciparum* gametocytes (samples 4 and 8) and *P. malariae* parasites (samples 1, 2, 3, 7, 8, and 10) during the period under study. Blood samples were collected on the dates indicated in the tabular portion of the figure, which summarizes the results from the typing of the merozoite surface antigen-1 (MSA-1) fragments amplified using primers A + B and of the MSA-2 fragments obtained using primers 2 + 3 and thrombospondin related anonymous protein (TRAP). troph. indicates parasitemia, expressed as the number of trophozoites/100 leukocytes. The size of the fragments was estimated using Boehringer Mannheim molecular weight marker VI. The allelic type assigned to the various fragments detected is indicated, as deduced from hybridization using family-specific probes under conditions of distinct stringency, as described in the Materials and Methods. The various alleles observed are indicated in the same order for size and allelic type. Hybrid genes are indicated in parentheses. The TRAP typing was done using size polymorphism and *Taq* I restriction fragment length polymorphism. The various TRAP profiles have been assigned arbitrary codes, a-d. bp = basepairs; ? = ambiguous typing results (unassigned allele), − = no fragment observed.



DNA	date	troph.	MSA-1 A+B	allele	MSA-2 1+4 size (bp)	allele
1	4/06/90	220	470+440+380+360	MAD20, K1, MAD20	760+710	3D7, FC27
2	22/06/90	0	440+380	MAD20, K1	-	-
3-A	30/06/90	1027	440+380	MAD20, K1	680	FC27
4	9/07/90	0	380	K1	-	-
5	24/07/09	120	440+380+360	MAD20, K1	760	3D7
6-B	30/07/90	400	440+380	MAD20, K1	760	3D7
7	15/08/90	0	410+380	K1, K1	-	-
8	24/08/90	37	410+380	K1,K1	680	3D7
9	7/09/90	294	380+290	K1, MAD20	680+620	3D7, FC27

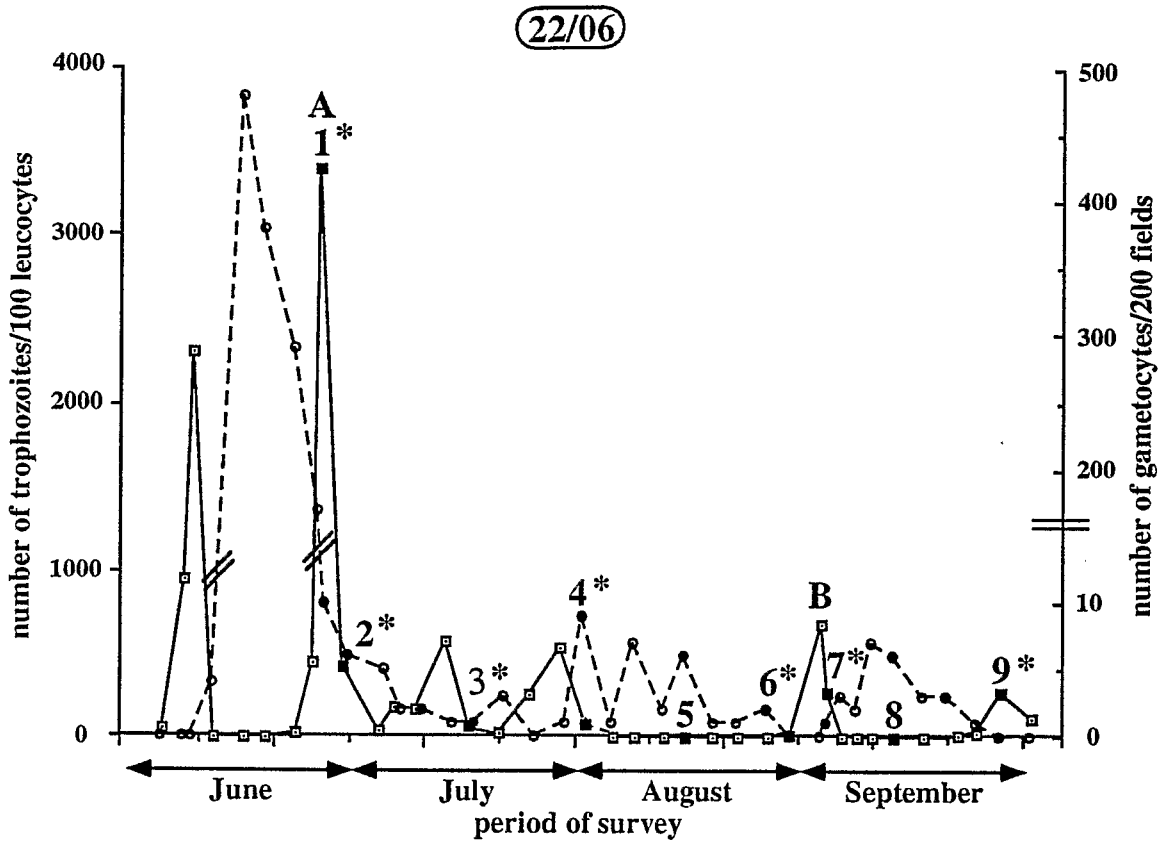
FIGURE 5. Course of *Plasmodium falciparum* parasitemia and typing of the parasites from child 02/03, a four-year-old girl, who experienced two clinical episodes that were drug-treated as described in the Materials and Methods. The child carried *P. falciparum* trophozoites (troph.) and *P. falciparum* gametocytes (**dotted line**), as indicated. In addition, a low density of *P. malariae* parasites was observed in sample 1. Note that the scales used for trophozoites and gametocytes differ. Nine blood samples, numbered 1–9, were collected on the dates indicated. For definitions of the symbols used, see Figure 1. Results of amplification of merozoite surface antigen-1 (MSA-1) using primers A + B and MSA-2 using primers 1 + 4 are shown in I and II, respectively. The numbers above each lane refer to the number of the DNA analyzed. 0 = no DNA added in the reaction; + = positive control (DNA from clone 89F5).[18] Genotyping is also indicated. Parasitemia is expressed as the number of trophozoites/100 leukocytes. For size determination and allele assignment, see Figure 3.

MAD20-type, identical to the one detected from DNA 1, collected during the asymptomatic period preceding episode A. While typing indicated that the parasite populations present during episodes A and B were different, the results suggested that in both episodes, the parasites causing the pathology were those carrying the 470-bp K1 type. This prompted us to analyze the other symptomatic episodes in the context of the preceding asymptomatic carriage.

Comparison of parasites carried before and during clinical attacks. The parasites carried by some children in the asymptomatic period preceding an attack were compared with those collected during the clinical episode. In addition to episodes A and B of child 14/07 noted above, this was also carried out for several episodes in three other children.

The parasites from the three episodes experienced by child 28/06 were compared with those collected in the preceding periods. As shown in Figure 4, DNA 2 yielded two distinct FC27-type MSA-2 fragments of 840 bp and 780 bp. From DNA 3, collected five days later during episode A, a single 840-bp MSA-2 band was generated, suggesting that the peak parasitemia was due to multiplication of this parasite type that became dominant. In contrast, DNA 7, collected during episode B, was more complex than DNA 6, collected nine days earlier. The B clinical attack was associated with the detection of three MSA-1 and three MSA-2 fragments. Staining agarose gels with ethidium bromide indicated that this parasite population contained a mixture of (at least) three distinct types present in a similar proportion. Two MSA-1 alleles (a 460-bp K1-type and a 350-bp, MAD 20-type) amplified from DNA 7 were not observed in the previous sample. Conversely, the 480 bp K1-type allele observed in sample 6 was not detected in DNA 7. Similarly, the 940-bp and the 870-bp 3D7/FC27 hybrids amplified from DNA 6 were no longer observed among the products obtained from DNA 7, while two new alleles, (a 940-bp 3D7-type and a 820-bp FC27-type), were generated from DNA 7. This indicates that the parasite population collected at the time of the B clinical attack contained novel parasite genotypes, but did not contain a single dominant type. Similarly, the patterns generated from DNA 10, collected during episode C, were more complex than those of the sample collected 12 days earlier, at a time when the child was asymptomatic (DNA 9). The DNA from episode C generated, in addition to the single MSA-1 or MSA-2 allele observed in DNA 9, two MSA-1 alleles (a 410-bp K1/MAD 20 hybrid and a 350-bp MAD20 allele), and one additional 920-bp 3D7-FC27 hybrid MSA-2 allele. In both reactions, however, the intensities of the ethidium bromide staining differed, the prominent bands being the 460-bp K1 MSA-1 allele and the 920-bp MSA-2 hybrid, suggesting that there was a single dominant type in DNA 10.

Figure 5 shows the course of parasitemia in child 02/03, a four-year-old girl, who experienced two malaria episodes associated with symptoms and high parasitemia, designated A and B. The other peaks of parasitemia were not febrile and decreased without treatment. The child carried *P. falciparum* gametocytes most of the time. All samples used for DNA preparation contained gametocytes, which complicated interpretation of the typing. Results obtained using MSA-1 and MSA-2 1 + 4 primers are shown in Figure 5, I and II, respectively. A 380-bp K1-type MSA-1 fragment was observed in all samples, including those collected after Quinimax® treatment, in which only a few gametocytes and no asexual forms could be detected. This fragment is, therefore, interpreted as being derived from the same population of



DNA	date	troph.	MSA1 A+B size (bp)	MSA1 A+B allele	MSA-2 1+4 size (bp)	MSA-2 1+4 allele	TRAP profile
1-A	27/06/90	3400	440	MAD20	900+830	3D7, 3D7	a
2	30/06/90	420	440	MAD20	900+830	3D7, 3D7	a
3	17/07/90	65	440+410+390	MAD20, K1, RO33	900	(3d7, FC27)	?
4	31/07/90	115	440+410+390	MAD20, K1, RO33	900	(3d7, FC27)	b
5	14/08/90	0	410	(K1, RO33)	-	-	-
6	28/08/90	37	410+390	(K1, RO33)	900	(3d7, FC27)	x
7-B	2/09/90	270	410+390	(K1,RO33)	900	(3d7, FC27)	b
8	11/09/90	0	-	-	-	-	-
9	25/09/90	265	440+410	MAD20, (K1, RO33)	900	(3d7,FC27)	x

FIGURE 6. Course of *Plasmodium falciparum* parasitemia and typing of the parasites from child 22/06, a two-year-old girl, who experienced two clinical episodes, designated A and B, which were drug-treated as described in the Materials and Methods. The child carried *P. falciparum* trophozoites and *P. falciparum* gametocytes **(dotted line)**, as indicated. Note that the scales used for trophozoites and gametocytes differ. For definitions of the symbols used, see Figure 1. Nine blood samples, numbered 1–9, were collected on the dates indicated. Typing results are summarized in the tabular portion of the figure. troph. refers to the number of trophozoites/100 leukocytes. bp = basepairs. TRAP = thrombospondin related anonymous protein. For size determination and allele assignment, see Figure 3.

TABLE 1

Genotyping of *Plasmodium falciparum* parasites from successive clinical episodes in 10 children*

Child	Age (years)	Clinical episode	Date	*P. falciparum* trophozoites†	MSA-1 A + B size	MSA-1 block 2 allelic family	MSA-2 1 + 4 size	MSA-2 allelic family	TRAP profile
02/04	1.5	A	7/26/90	648	520 + 420	K1, K1	880 + 820	FC27, FC27	a
		B	9/29/90	732	560	MAD20	800	3D7	b
14/05	6	A	6/20/90	1,500	470 + 380	x, x	780 + 740	FC27, 3D7	a
		B	7/11/90	1,300	410	x	740	FC27	b
		C	8/6/90	630	490	K1	920	x	c
		D	9/1/90	584	490	K1	920	FC27, 3D7, 3D7	c
19/09	3	A	7/3/90	1,600	440 + 410 + 380	(K1, MAD20), MAD20, ?	870	(3D7, FC27)	a
		B	7/25/90	500	440 + 410 + 380	(K1, MAD20), MAD20, ?	980 + 840	3D7, (3D7, FC27)	b
		C	8/19/90	400	480 + 460 + 440	?, ?, K1	820	(3D7, FC27)	c
14/14	4	A	6/22/90	370	430	MAD20	920	3D7	a
		B	7/8/90	1,500	410 + 360 + 310	K1, MAD20, RO33	920 + 750	3D7, FC27	b
		C	7/28/90	1,500	430 + 310 + 290	K1, MAD20, MAD20	780	(3D7, FC27)	c
		D	8/20/90	500	430	(MAD20, K1)	780	3D7	d
28/06	6	A	7/4/90	729	430	K1	840	FC27	a
		B	8/15/90	236	460 + 410 + 350	K1, (K1, MAD20), MAD20	940 + 820 + 780	3D7, FC27, FC27	c
		C	9/15/90	1,100	460 + 410 + 350	K1, (K1, MAD20), MAD20	920 + 840	(3D7, FC27), FC27	d
02/03	4	A	6/30/90	1,027	440 + 380	MAD20, K1	680	FC27	NA
		B	7/30/90	400	440 + 380	MAD20, K1	760	3D7	NA
12/07	1	A	6/23/90	663	480 + 420 + 400	(MAD20, K1), RO33, (MAD20, K1)	940 + 800	FC27, FC27	a
		B	7/5/90	655	480 + 420 + 400	(MAD20, K1), RO33, (MAD20, K1)	840 + 800	FC27, FC27	b
22/06	2	A	6/27/90	3,400	440	MAD20	900 + 830	3D7, 3D7	a
		B	9/2/90	270	410 + 390	(K1, RO33)	900	3D7, FC27	b
08/03	5	A	6/30/90	260	390	(K1, RO33)	880	FC27	a
		B	7/16/90	246	390	(K1, RO33)	880	FC27	a
14/07	2	A	6/27/90	830	470 + 420	K1, MAD20	580	3D7	NA
		B	7/3/90	1,180	470	K1	580	3D7	a
		C	7/23/90	1,046	570 + 520 + 470	K1	580 doublet	3D7, FC27	NA
		D	9/22/90	1,306	540	MAD20	650 + 450	FC27, FC27	b

* Episodes were arbitrarily coded A–D for each child. The size of the fragment is expressed in basepairs. The assignment to a specific allelic family was based on the results of hybridizaiton using allele-specific probes as described in the Materials and Methods. Hybrid genes (intragenic recombinants) hybridized with two distinct probes under nonstringent conditions and failed to hybridize with any of the probes at high stringency. They are indicated in parentheses. MSA-1 = merozoite surface antigen-1. x = alleles that failed to hybridize under nonstringent conditions. ? = unassigned alleles due to ambiguous hybridization results. (Sequencing indicated that they are complex mosaic forms of previously described allelic types). The thrombospondin related anonymous protein (TRAP) typing was done as described in the Materials and Methods. Arbitrary codes (a–d) have been given for each series of samples analyzed from a child. NA = not available.

† Values are the number of trophozoites/100 leukocytes.

gametocytes carried throughout the period under study. No specific MSA-2 allele could be associated with these gametocytes, probably because of insufficient sensitivity of the detection. The genetic characteristics of the parasites carried asymptomatically before each clinical episode could be compared with those of the parasites collected during the episode. Sample DNA 3, collected during episode A, clearly differed from DNA 1. Interestingly, a 440-bp MAD 20-type MSA-1 allele, barely visible in the products obtained from DNA 2 as shown in Figure 5, gave a strong dominant signal in DNA 3, suggesting that the parasites present at high density and causing episode A were present in low amounts (below the level of microscopic detection) eight days before. This is consistent with a $> 10^3$ increase in parasite density observed within that period. Sample DNA 6, collected during episode B, differed from DNA 5 collected six days earlier, in presenting a less complex picture: a 360-bp K1-type MSA-1 fragment present in DNA 5 could not be detected in DNA 6, suggesting that again the parasites causing episode B contained a dominant type. An additional peak of parasitemia (DNA 9), not febrile but associated with headache, also corresponded with the presence of parasites that had not been detected before, characterized by a 290-bp MAD 20-type MSA-1 allele and a 620-bp FC27-type MSA-2 allele. In this child, the peaks of parasitemia, whether febrile or not, were therefore associated with detection in the peripheral circulation of novel genotypes, distinct from those observed during asymptomatic carriage.

The detection of novel genotypes during one episode was also observed in the analysis of parasites from child 22/06. The course of parasitemia in this two-year-old child, who also carried gametocytes most of the time, and typing of the parasites are shown in Figure 6. Three DNA samples (3, 4, and 5) were collected during the period spanning from episode A (DNAs 1 and 2) to episode B (DNA 7). Both DNAs 1 and 2 generated a 440-bp MAD 20 MSA-1 allele and 900-bp and 830-bp MSA-2 alleles of the 3D7-type in similar proportions, suggesting that this sample contained a mixture of two parasite types presenting indistinguishable MSA-1 alleles. Sample DNAs 3 and 4 yielded identical MSA-1 profiles and the same MSA-2 allele, whereas DNA 5, collected from a sample containing gametocytes and no microscopically visible trophozoites, contained a single MSA-1 allele typed as a 410-bp K1-RO33 hybrid. No MSA-2 allele could be amplified from this sample, probably due to low parasite density. Sample DNA 6, collected four days before episode B, generated the same pattern as DNA 7, collected during episode B: 410-bp and a 390-bp K1-RO33 MSA-1 hybrids. The 390-bp fragment was more abundant in the products amplified from DNA 7 than in those generated from DNA 6, suggesting that episode B was caused by the parasites that carried this allele.

DISCUSSION

Data described in this report as well as in other molecular studies of parasites from Dielmo[10, 14, 15] indicate that genetic diversity of *P. falciparum* parasites circulating in this village is quite large. So far, the only cases in which identical PCR patterns have been observed were DNA samples collected a few days apart during the same clinical episode or during a recrudescence resulting from incomplete parasite clearance after treatment of the first episode, i.e., cases where the parasites are predicted to present identical genetic characteristics. This indicates that the technique used is reliable both qualitatively and quantitatively and that distinct PCR patterns do indeed reflect intrinsic genetic differences. The PCR typing approach used does not allow determination of the karyotype, and thus we do not know the actual number of distinct clones present in the isolates studied nor the precise gene combination of each of these clones. However, the results unequivocally show that the parasite populations differ in the majority of isolates analyzed so far. All major allelic forms of the MSA-1 and MSA-2 genes described in the literature (for a review, see the report by Kemp and others[6]) have been detected in the village. In the present study, 34 distinct MSA-1 alleles and 31 distinct MSA-2 alleles were observed. In a cross-sectional study of asymptomatic carriers, carried out using samples collected during the 1992 transmission season, 23 distinct MSA-2 alleles have been detected.[14] These are minimal estimates, since DNA sequencing is likely to detect additional differences.

In Dielmo, clinical malaria could be associated with parasite densities above an age-dependent threshold, determined as 275 trophozoites/100 leukocytes at one year of age, decreasing to 191 trophozoites/100 leukocytes by the age of six years.[16] In all cases investigated, such levels were reached after a very rapid increase in parasite density, suggesting unrestrained parasite growth. The genetic characteristics of the parasite populations collected during a malaria episode differed from those detected in the blood sample collected in the preceding period. The PCR profiles were either less complex, suggesting outgrowth of a dominant population or inversely more complex, indicating the presence of additional parasite alleles and thus of additional parasite types. Whether these are dominant is difficult to determine in complex allelic mixtures.[10] Overall, however, the complexity of the infections, reflected by the number of distinct alleles detected, was lower during the clinical episodes than during the phases of asymptomatic carriage. The mean number of MSA-2 alleles detected here in DNAs collected from clinical episodes was 1.4 per sample, while it was 4 in DNAs collected from children who remained asymptomatic during a similarly high transmission season.[14] This is not a general rule because complex populations have also been observed in samples collected during a malaria episode.[10] The trend is, nevertheless, clearly for less complex infections during a clinical episode than during asymptomatic carriage. We interpret this as indicating that a dominant population is, in fact, responsible for the observed clinical event.

Comparison of the genotypes of the parasites collected during the successive clinical episodes experienced by a child showed that the parasite populations collected from the peripheral circulation were different for each episode. As summarized in Table 2, this was observed in three children, who experienced two episodes, in two children who had three clinical attacks, and in three children who had four clinical malaria episodes during this four-month period of intense transmission. The single case in which the genotypes of two successive episodes could not be distinguished using the four typing reactions used here was interpreted as a recrudescence. Since each episode was treated with quinine,

TABLE 2

Comparison of the merozoite surface antigen-1 (MSA-1), MSA-2, and thrombospondin related anonymous protein (TRAP) polymerase chain reaction patterns of the parasite population collected during successive clinical episodes in 10 children. The specific patterns (number of bands, size of the fragment, and allelic type) obtained for the parasite population collected during successive clinical episodes, coded A–D, have been compared*

Child	MSA-1 block 2	MSA-2 central domain	TRAP central domain
02/04	A ≠ B	A ≠ B	A ≠ B
14/05	A ≠ B ≠ C = D	A ≠ B ≠ C ≠ D	A ≠ B ≠ C = D
19/09	A = B ≠ C	A ≠ B ≠ C	A ≠ B ≠ C
14/14	A ≠ B ≠ C ≠ D	A ≠ B ≠ C ≠ D	A ≠ B ≠ C ≠ D
28/06	A ≠ B = C	A ≠ B ≠ C	NA
02/03	A = B	A ≠ B	NA
12/07	A = B	A ≠ B	A ≠ B
22/06	A ≠ B	A ≠ B	A ≠ B
08/03	A = B	A = B	A = B
14/07	A ≠ B ≠ C ≠ D	A ≠ B ≠ C ≠ D	B ≠ D

* A, B, C, and D are arbitrary codes for the successive clinical epidodes in each child. NA = not available.

rapidly resulting in a negative blood smear and disappearance of the alleles identified during the clinical attack, long-term carriage of parasites was precluded in these children. This indicates that clinical malaria episodes were induced by recently inoculated parasites. During the period under study, the mean inoculation rate was one infective bite every second night. With such a large local parasite diversity, inoculation of new strains should have been a frequent event. A longitudinal analysis of the strains harbored by children who remained asymptomatic (and thus untreated) during the same period showed successive waves of genetically distinct parasites.[15] This rapid turnover of strains in the peripheral circulation was consistent with a frequent inoculation of new parasite populations. It is noteworthy that even under such an intense transmission, some children remained apparently free of parasites for long periods (up to two months), presenting negative blood smears and no evidence of parasites by PCR (neither by direct examination of the PCR product nor by hybridization). We do not know whether this absence of parasites indicates that these children did not receive any infective bite for several weeks or whether they had the capacity to rapidly clear many strains, while still being fully susceptible to other ones.

The genotypes of the parasites present in the peripheral circulation during the clinical episodes analyzed here were all different. This suggests that there is no precise pathogenic type, but rather that clinical attacks may be caused by a large number of distinct strains. For some children, the encounter with *P. falciparum* parasites had a different outcome for different strains. Indeed, one of the most important observations was that in the children presenting alternating periods of symptomatic or asymptomatic carriage, the genetic characteristics of the parasites carried at low density during asymptomatic phases differed from those of the parasites causing a clinical episode, which quickly reached high parasitemia. This indicates that the factors controlling parasite density in these children were strain-specific. This could reflect differences in the growth rate of various strains, resulting from the poor fitness of some types in certain hosts, such as, for instance, poor invasion efficiency, impaired intraerythrocytic maturation, or a slow replication rate, and/or reflect specific immune pressure, which would restrict parasite multiplication of certain strains while leaving other strains unaffected. The hypothesis that strain-specific immune responses participate in control of parasite density in semi-immune children is in agreement with the conclusions drawn from experimental infections in both primates[17] and humans.[4,5] This does not preclude the possibility that maturation of the immune response elicited by a specific strain and/or exposure to additional strains subsequently results in protective mechanisms transcending strain specificities. Elucidation of the respective contribution of strain-specific and nonspecific responses to control of parasite propagation is a key element in our understanding of protection against malaria. To date, this has been difficult to study because the immune effectors contributing to parasite clearance in humans are unclear, and as a consequence, the target antigens of protective immune mechanisms are still to be determined. Several antigens presenting considerable serologic diversity have been described. The variant antigen located on the surface of the infected red blood cell elicits variant-specific immune responses.[18–20] While the contribution of such an antibody to parasite clearance remains to be demonstrated in humans, protection was shown to be variant-specific in *Saimiri* monkeys.[20] The large local allelic polymorphism of the MSA-1 and MSA-2 genes observed here and in a few other studies[21,22] raises the interesting alternative that these major merozoite antigens[23,24] could constitute strain-specific targets. Investigation of the immune response of the children studied here to various allelic forms of these antigens is underway.

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