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PCR Typing of Field Isolates of *Plasmodium falciparum*

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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 TaqI 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 *Anopheles* 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, allelic 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, 35, 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.
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/CBPalo Alto strain was cultivated as described by Fandeure et al. (10), and highly synchronous parasites were obtained by plasmagelflotation and the sorbitollysis 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 H₂O.

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 TGAC-3’) and MSA-1B (5’-ATTCATTAATTTCTTCATATCCATC-3’). For the MSA-2 gene the following primers were used: MSA-2 1 (5’-ATGAGTTAATTAAAACATTGTCTATTATA-3’) and MSA-2 2 (5’-AACGAATTCATAAACAAATGCTTATAATATGAGT-3’). For the amplification of the TRAP gene, the following primers were used: TRAP 3 (5’-ATGGATATTTGCTGATTCTGCATTGGA-3’) and TRAP 4 (5’-TATCTTCACTATTAGTACGTGCCTATTTCC-3’). These sequence of the primers used to amplify the central region of the CS gene were 5’AGAGATGGAAATAACGAGACAACGAG-3’ (CS3) and 5’-GTCATTTGGCATTAAGTGACCTTGCTCC-3’ (CS4).

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 TaqI DNA polymerase (Promega, Charbonnières, France) in the buffer supplied by Promega (12 mM MgCl₂). 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

<table>
<thead>
<tr>
<th>Parasite DNA</th>
<th>Allelic type used as reference for:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 89F5</td>
<td>MSA-1 A–MSA-1 B</td>
</tr>
<tr>
<td>Clone Ig2G1</td>
<td>K1</td>
</tr>
<tr>
<td>Senegal 2</td>
<td>MSA-1 H–MSA-1 I</td>
</tr>
<tr>
<td>Senegal 4</td>
<td>MSA-2 2–MSA-2 3</td>
</tr>
<tr>
<td>Senegal 5</td>
<td>INDO/3D7</td>
</tr>
</tbody>
</table>

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 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 (20). Probes were prepared by nick translation, with a nick translation kit from Boehringer Mannheim (SeaKem GTG; Tebu). Assignment of MSA-1 and MSA-2 to specific allelic families was done by hybridizing Southern blots with allele-specific probes (20). Probes were prepared by nick translation, with a nick translation kit from Boehringer Mannheim (SeaKem GTG; Tebu).

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 amplification of each marker were analyzed with the DNA from clone 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 with ethidium bromide staining, which, within a certain range, gives a good estimate of the DNA concentration.
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
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 HinI1 and BglII sites has been reported previously (29) and is depicted in Fig. 1. In the region amplified in the present study, HinI1 and BglII RFLPs were indeed observed (data not shown), but were less informative than TaqI restriction. As shown in Fig. 5B, the TaqI 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
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 AvrII or HpaII RFLP (22) were unsuccessful. The complexity of the allelic mixtures generated multiple band profiles that did not permit allele assignments.

**DISCUSSION**

Parasite typing is a 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 TaqI on its own discriminated the nine isolates. This is interesting because limited TaqI 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.
for MSA-1 and MSA-2, which gave multiple band patterns specific for each sample. With the exception of one MSA-1 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 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.

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