PCR Characterization of Isolates from Various Endemic Areas: Diversity and Turn Over of *Plasmodium falciparum* Populations are Correlated with Transmission

Pierre Daubersies, Selma Sallenave-Sales /*****, Jean François Trape*, Lucie Raharimalala**, Christophe Rogier***, Hughes Contamin****, Thierry Fandeur****, Claudio T Daniel-Ribeiro*****, Odile Mercereau-Puijalon*, Pierre Druilhe /+

Laboratory of Biomedical Parasitology Institut Pasteur, Paris *ORSTOM, BP 1386 Dakar **Pasteur Institute, Antananarivo, Madagascar ***Pasteur Institute, BP 220, Dakar, Senegal ****Unit of Experimental Parasitology, Pasteur Institute, 75724 Paris Cedex 15, France *****Departamento de Imunologia, Instituto Oswaldo Cruz, 21045-900 Rio de Janeiro, Brasil

Key words: malaria - *Plasmodium falciparum* - PCR - MSA1 - MSA2 - characterization

We have previously investigated the extend of genetic diversity within *Plasmodium falciparum* isolates by using an improved cultivation technique for the cloning of parasites (Mazier et al. 1984) and further characterizing those by 4 distinct typing techniques (Druilhe et al. in preparation). Two series of 33 clones derived from 2 isolates obtained in primary attack cases were produced by cloning on the day of sampling and cultivating them together with hepatocytes feeder layers, a method which was found able to promote the growth of parasites not adapted to culture conditions. Drug response and karyotype analysis demonstrated a large degree of diversity among these clones. Restriction Fragment Length Polymorphism, revealed by probing with the pPFrep20 sequence, also confirmed the above results, however showed a high degree of genetic relatedness of the clones, which was unexpected from the results of the other techniques. Finally, amplification by polymerase chain reaction (PCR) of a polymorphic region of 4 genes (see below) revealed respectively 6 and 4 patterns of allelic forms in 24 clones for each isolate.

Therefore, due to genetic relatedness of the clones, PCR did not prove to be the most discriminating technique. However, in view of its great sensitivity, it has the major advantage of being able to reveal the co-existence of distinct parasite populations in an isolate without the need to cultivate parasites in order to produce enough DNA.

+ Corresponding author

For this reason, PCR was employed in further epidemiological studies. Using primers derived from molecules which are considered as valuable vaccine candidates, we investigated the genetic diversity of parasites harbored by acute or chronic *P. falciparum* carriers in various areas of the world which differed by the rate of malaria transmission by mosquitoes, and also in a longitudinal manner in some patients.

RESULTS

The PCR technique used here was based on amplification of highly polymorphic regions from two markers: block 2 of the Merozoite Surface Antigen 1 (MSA1) (Tanable et al. 1987) and the repeated region of the Merozoite Surface Antigen 2 (MSA2) (Smythe et al. 1990) according to a procedure described in more details elsewhere (Contamin et al. in preparation; see legend to Fig. 1). In all cases, specificity of the PCR products was confirmed by hybridization with a mixture of probes (data not shown) corresponding to the various alleles already described for these two genes (Tanabe et al. 1987, Certa et al. 1988, Smythe et al. 1990).

PCR was first employed with 2 series of samples originating from relatively low endemicity and low transmission rate areas: 12 symptomatic adults collected between February and April 1993 in the High Lands of Madagascar (Fig. 1A) and 20 symptomatic Brazilian adults collected in 1990 and 1992 in the State of Rondonia (Amazonia) (Fig. 1B). By combining the two markers, every sample gave

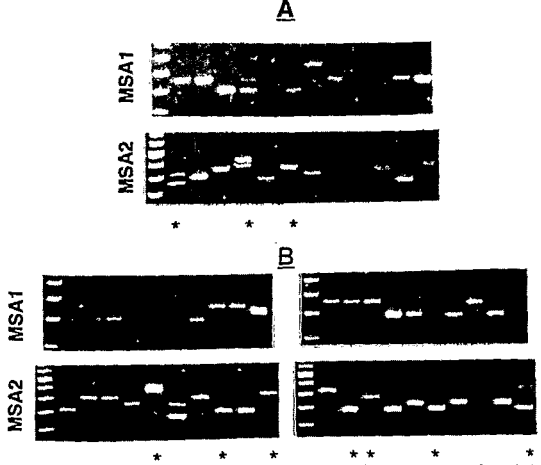

Fig. 1: PCR analysis of the polymorphic regions of MSA1 (block 2) and MSA2 (repeated region) form 12 Malagasi (A) and 20 Brazilian (B) isolates of *Plasmodium falciparum*. Asterisks (*) correspond to mixed infections. DNA from approximately 5.10^7 red blood cell were extracted as previously described (Fandeur & Mercereau-Puijalon, 1991) and amplified according to manufacturer recommendations (Amersham) in an automated heatblock (Hybaid) for 35 cycles (2", 94°C; 1', 55 °C; 2', 72 °C). Sequence of MSA1 primers was: 5'-AAGCTTTAGAAGATGCAGTATTGAC-3' and 5'-ATTCATTAATTTCTTCATATCCATC-3'; sequence of MSA2 primers was reported in Fandeur & Mercereau-Puijalon, 1991. Ten µl of PCR reaction were loaded on a 3% NuSieve 3:1 agarose gel (FMC Bioproduct, USA) containing ethidium bromide. DNA size markers were 100bp ladders for BRL. Gels were blotted to Nylon membranes (Hybond-N, Amersham) and simultaneously hybridized with probes corresponding to K1, MAD20 and RO33 alleles for MSA1 or IND/3D7 and FC27 alleles for MSA2 (data not shown).

a distinct pattern, indicating a large degree of polymorphism in these regions and also confirming the efficiency of PCR as a tool for characterizing parasite populations within isolates. In 3/12 samples from Madagascar (25%), 2 distinct amplified bands were detected, indicating that these individuals were infected by several parasite populations. In the Brazilian series, 7/20 individuals (35%) presented mixed infections. In a previous study also based on MSA1 amplification of block 2 by PCR, Kimura et al. (1990) observed only 20% of mixed infections among Brazilian patients, which may be due to the distinct geographical origin of the samples or to a higher sensitivity of the PCR protocol used here.

In order to further investigate the correlation between the degree of polymorphism observed by PCR and the rate of transmission by mosquitoes, we performed follow-up studies in two regions differing clearly by the epidemiology of the disease, that is i) an holoendemic area and ii) an urban area during a non-transmission period. The latter study was carried out in the district of Pikine, a suburb of Dakar (Senegal), where 3 asymptomatic chronic carriers were sampled every 1-2 weeks over a period of 5 weeks. At the time of collection (Jan.-Feb.1993), the transmission was totally stopped (Trape 1993). Fig. 2 shows a remarkably simple and stable PCR pattern for each of the 3 individuals, with patients A and B presenting identical profiles for both markers. Variations in the intensity of the ethidium bromide stained bands are difficult to interpret as we did not perform a quantitative PCR. Thus it appears that in low transmission areas, during a non-transmission period, the parasites harbored by the individuals are homogeneous and importantly remain genetically stable at least for the markers studied.

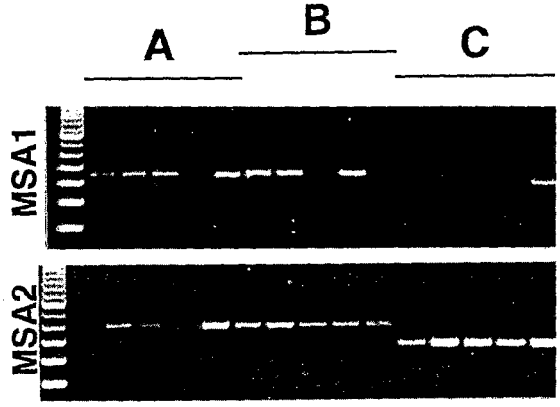

Fig. 2: PCR analysis of the polymorphic regions of MSA1 (block 2) and MSA2 (repeated region) from Senegalese isolates of *P. falciparum* sampled in the district of Pikine. Patients A, B and C were collected every 1-2 weeks over a period of 5 weeks at days 1, 15, 22, 29 and 36, from January to February 1993. DNA size markers were 100 bp ladders for BRL.

Interestingly, these results were in deep contrast with those obtained in the second follow-up study carried out in the village of Dielmo (Senegal), an holoendemic area where the transmission rate at the time of sampling (June to September 1992) was estimated between 10 and 30 infected bites per individual and per month (Trape JF unpublished data). For this study, 4 asymptomatic individuals, three 7- and 8-year-old children (indiv. A, B, C) and one protected 26-year-old adult (Indiv. D), were sampled 4 times at 2-4 weeks intervals. Figure 3 shows the extremely high diversity observed within each sampled and for both markers. Up to 6 bands corresponding to at least 6 parasite populations could be visualized in some samples (A1 and B2) on agarose gel. Furthermore, for all these individuals and for both markers, a different PCR pattern was consistently observed between 2 succesive sam-

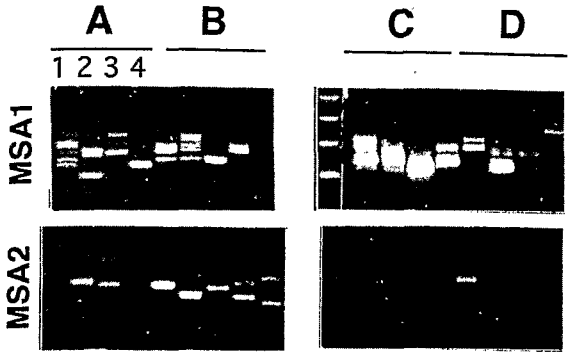

Fig. 3: PCR analysis of the polymorphic regions of MSA1 (block 2) and MSA2 (repeated region) from Senegalese isolates of *P. falciparum* sampled in the village of Dielmo. Patients A, B and C were asymptomatic 7- and 8-year-old children; patient D was an asymptomatic 26-year-old adult. Four samples (1, 2, 3, 4) were collected to intervals of 2-4 weeks from June to September 1992 (rainy season). DNA size markers were 100bp ladders from BRL.

plings, that is within 2-4 weeks time, indicating a very fast renewal of parasite populations in a given individual.

In order to estimate how fast could be the turnover of parasite populations in this area, a complementary follow-up study was carried out on 3 other asymptomatic individuals, 2 adults (indiv. A, B) and a 16-year-old teenager (indiv. C), who were sampled (fingerpricks specimens) every 2-4 days (Fig. 4). During this follow-up, some of the samples

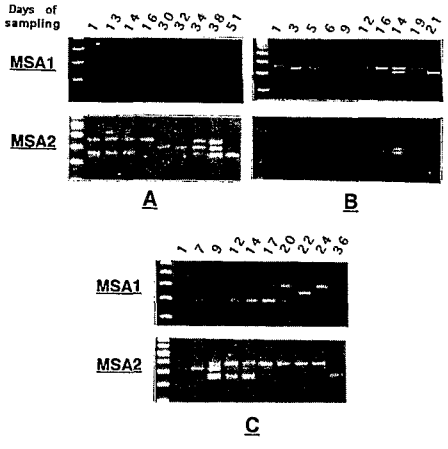

Fig. 4: PCR analysis of the polymorphic regions of MSA1 (block 2) and MSA2 (repeated region) from Senegalese isolates of *P. falciparum* sampled in the village of Dielmo. Patients A and B were asymptomatic 49- and 52-year-old adults; patient D was an asymptomatic 16-year-old teenager. Samples were taken every 2-4 days from August to September; Samples giving no amplification were omitted. DNA size markers were 100bp ladders from BRL.

were negative and could not be amplified by PCR (indiv. A and C). Strikingly, some PCR patterns appeared to change within a period of time as short as 2 days.

DISCUSSION

Despite its limitations in discriminating genetically related *P. falciparum* clones, the analysis by PCR of parasite polymorphism described here clearly support the potential of this technique for the characterization of plasmodial isolates. Indeed, the identification of subpopulations within isolates infecting a single individual in longitudinal studies permits new insights in the behavior of parasite populations according to various parameters such as the immune status of the infected individuals or the local transmission rate of the disease. Even within the context of this limited study, this approach provides novel information on the relationships between distinct individual parasites within a single human host.

Comparison of PCR patterns obtained with isolates from several parts of the world and analyzed in similar experimental conditions indicates that there is some correlation between the degree of diversity observed within an isolate and the transmission rate of the studied region. As expected a high degree of polymorphism was observed between isolates collected in the same area. The amplified fragments exhibited a wide range of sizes with both markers in the African and the Brazilian samples. Similar results have been previously reported by others using the same technique (Kimura et al. 1990, Merceau-Puijalon et al. 1991, Scherf et al. 1991, Snewin et al. 1991) or antibody typing (McBride et al. 1982). Amplification of different MSA1 and MSA2 alleles indicated the coexistence of multiple parasite genotypes within an isolate. The comparison of complex PCR patterns in Dielmo samples with more simple patterns in Brazil, Madagascar and Pikine suggested that the complexity of the infections could be related to the level of transmission by mosquitoes.

Somewhat unexpectedly, no relationship was observed between the complexity of the infections and the immune status of the individuals studied. Indeed, non-protected children from Dielmo (Fig. 3) had much more complex PCR patterns than non-protected adults from low endemicity areas (Figs 1, 2).

Results obtained in the 2 Senegalese foci demonstrated a further correlation between the turn-over rate of parasite populations within an individual and the transmission of new infections by mosquitoes. Indeed, in the Pikine study (Fig. 2) at a time of non-transmission, PCR patterns were simple and stable, indicating that parasite subpopulations defined by MSA1 and MSA2 markers did not change over time. In contrast to this situation, the results obtained in Dielmo (Figs 3, 4) demonstrate that in holoendemic areas, asymptomatic individuals can harbor very heterogeneous and constantly renewed populations with a turn-over period as short as 2-4 days in some individuals. Furthermore, taking into consideration the stability observed in Pikine in the absence of reinfection, it would appear that this fast turn-over is due to superinfection, i.e. to the transmission rate. This suggests that the fast emergence of parasites with distinct polymorphic markers recorded in the high transmission areas is due to the emergence in the blood of new parasite populations inoculated by infected mosquitoes. Does this mean that the previous populations are eliminated (by the immune system) or that they are not circulating any more in the peripheral blood? This important question needs further investigations.

The two polymorphic markers used here belong to the group of candidate molecules presently evaluated for vaccine purposes. If polymorphic molecules are indeed the targets of protective immunity, the results described here clearly show how important is the number of distinct alleles a vaccine will have to handle within a short time, in highly endemic areas.

REFERENCES

Certa U, Rotmann D, Matile H, Reber-Liske R 1988. A naturally occuring gene encoding the major surface antigen precursor p190 of *Plasmodium falciparum* lacks tripeptide triplets. *EMBO J 6:* 4137-4142.

Fandeur T, Mercereau-Puijalon O, 1991. *Plasmodium falciparum*: genetic stability of the Uganda Palo Alto strain propagated in the Squirell Monkey (Saimiri sciureus). *Exp Parasitol 72:* 223-235.

Kimura E, Mattei, D, Mana di Santi S, Scherf A 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.

Mazier D, Druilhe P, Guguen-Guillouzo C, Bayard P, Soeun V, Datry A, Gentilini M, 1984. Hepatocytes as feeder-layers for *in vitro* cultivation of *Plasmodium falciparum* blood-stages. *Trans R Soc Trop Med Hyg* 78: 330-334.

McBride JS, Walliker D, Morgan G 1982. Antigenic diversity in the human malaria parasite. *Science* 217: 254-257.

Merecereau-Puijalon O, Fandeur T, Bonnefoy S, Jacquemot C, Sarthou JL 1991. A study of the genomic diversity of *Plasmodium falciparum* in Senegal. 2. Typing by use of polymerase chain reaction. *Acta Trop 49:* 293-304.

Scherf A, Mattei D, Sarthou, JL 1991. Multiple infections unusual distribution of block 2 of the MSA1 gene of *Plasmodium falciparum* detected in west Africans clinical isolates by polymerase chain reaction analysis. *Mol. Biochem. Parasitol 44:* 297-299.

Smythe, JA, Peterson, MG, Coppel R, Saul AJ, Kemp DJ, Anders RF 1990. Structural diversity in the 45 kilodalton merozoite surface antigen of *Plasmodium falciparum. Mol Biochem Parasitol 39:* 227-234.

Snewin VA, Herrera M, Sánchez G, Scherf A, Langsley G, Herrera S 1991. Polymorphism of the alleles of the merozoite surface antigens MSA1 and MSA2 in *Plasmodium falciparum* wild isolates from Colombia. *Mol Biochem Parasitol 49:* 265-75.

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

Trape, JF, Lefebvre-Zante E, Legros F, Druilhe P, Rogier C, Bouganali H, Salem G 1993. Malaria morbidity among children exposed to low seasonal transmission in Dakar, Senegal and its implications for malaria control in Tropical Africa. *Am J Trop Med Hyg 48:* 748-756.