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Full Length Research Paper

Survival strategies of malaria episode, outcome and implications of treatment interventions

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Polymorphism and antigenic variation are important biological survival strategies of malaria parasites determining the episode, outcome and implications of treatment interventions. In *P. falciparum*, polymorphic antigens are associated with the asexual blood-stage; merozoite surface protein 2 (MSP2). The MSP2 genes have been invaluable in post-treatment discrimination of parasite resurgence from new infection, especially in high transmission areas. We performed polymerase chain reaction (PCR) on DNA extracted from blood samples of 141 malaria-infected infants, followed by restriction fragment length polymorphism (RFLP) of PCR products. The findings showed multiplicity of infections of single to six infections with an average of 2.58 infections per patient. Single infections of either 3D7 or FC27 allelic families of the MSP2 gene occurred in 51 patients (50.5%) out of all PCR- RFLP successful samples (n = 101). Out of 15 (10.6%) follow up samples with resurgent parasitaemia, 3 (20%) samples had recrudescent infections while 12 (80%) had variable results. Our findings provide an insight on the prevalence of the genetic determinants of suphadoxine-pyrimethamine (SP) resistance in Mlimba during the study period, and in the face of rapidly spreading resistance, calls for the periodic surveillance in order to timely detect early warning signal of the deteriorating SP cure rate.

Key words: Malaria, *Plasmodium falciparum*, MSP2 genes, Multiplicity of infections, Mlimba, Tanzania.

INTRODUCTION

Following widespread of chloroquine (CQ)-resistant *Plasmodium falciparum* strains, which evolved from early 1950s in the country, Tanzania changed and adopted the use of sulphadoxine - pyrimethamine (SP) as an interim first-line drug and amodiaquine (AQ) as second-line drug against uncomplicated malaria in August 2001. During the same period, SP was also approved as first or second-line antimalarial drug by most African countries for treatment of uncomplicated malaria (Sibley et al., 2001). The basis for complimentary SP use relies on its activity against CQ-resistant strains, ease of administration, tolerability and low cost compared to other available antimalarial drugs (Foster, 1994; Fevre and Barnish, 1999). However, subsequent reports of rapid spread of resistance against SP in Tanzania (Mutabingwa et al., 2001) and elsewhere in Africa (Jelinek et al., 2002; Kublin et al., 2002; Bwijo et al., 2003) has created a debate on its persistent use as first-line drug against malaria parasite in the region.

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Polymorphism and antigenic variation are common survival strategies of the malaria parasites in human populations (Felger et al., 1999). Individuals living in areas where *P. falciparum* is endemic experience numerous episodes of infection with symptoms and outcome varying depending on a combination of parasite factors (the structure of the parasite population and the survival strategies of the parasite) and host factors reflected by the immune response, and the implications of interventions (Hoffman et al., 2001). Polymorphic antigens have been described in several parasite life cycle stages but are particularly a feature of the antigens associated with the surface of the asexual blood-stage merozoites, merozoite surface protein 2, MSP2 (Scythe et al., 1990; Felger et al., 1999). These parasite surface proteins are polymorphic and have antigenic role to the parasite survival (Aubouy et al., 2003).

MSP2 is a 45- to 52 -kDa integral membrane glycoprotein encoded by a single-copy gene, and is anchored on the surface of the merozoite by a glycosylphosphatidylinositol (GPI) moiety. MSP2 sequences usually are assigned to one of two families, FC27 and IC-1/3D7, on the basis of the non-repetitive sequences (Fenton et al., 1991; Smythe et al., 1991; Snewin, 1991) . Sequencing of MSP2-alleles and PCR-RFLP from a large number of parasite isolates from different geographical locations has shown a virtually dimorphic structure of the molecule (Felger et al., 1999). This antigenic diversity of the parasites renders the highly polymorphic MSP2 gene suitable as a marker for genotyping of *P. falciparum* and enumerating multiple concurrent infections in blood samples and distinguishing individual alleles (Scythe et al., 1990; Felger et al., 1999; Aubouy et al., 2003).

Characterisation of the polymorphic MSP2 antigen has been used to establish whether a parasitaemia observed after treatment is caused by a recrudescence of resistant parasites or by a new infection (Babiker et al., 1994; Al-Yaman et al., 1997). This could be helpful in *in vivo* tests, particularly in areas with a considerable amount of transmission where, after a certain time, it is impossible to distinguish between recrudescence and new infection. Genotyping of field isolates is useful in detecting not only the multiplicity of mixed clone infections, but also aids in studying infection dynamics and finding markers for virulence and drug resistance (Felger et al., 1999). Genotyping of the MSP2 gene is achieved by a combination of Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) (PCR-RFLP) analyses. Using PCR-RFLP approach, Babiker et al. (1994) and Hill and Babiker (1995) showed a multiplicity of infections ranging between 1 – 6 parasite clones per infection in patients of Michenga, an area about 155 km east of Mlimba along Kilombero valley in Tanzania. In the present study we use PCR-RFLP approach to determine the multiplicity of infections concurrently present in the patient living in *P. falciparum*-endemic area of Mlimba division of Kilombero district in Morogoro, Tanzania.

MATERIALS AND METHODS

Study area

The study was conducted at Ifakara Health Research and Development Center (IHRDC) situated at Ifakara Town in Kilombero District, Morogoro, Tanzania. Samples used in this study were collected by the IHRDC from Mlimba, an area about 150 km away, south-east of Ifakara. Mlimba lies along Kilombero River where *falciparum* malaria is endemic with perennial transmission. This area is among nine sentinel sites for National Malaria Control Program in Tanzania since 1997 and its human population dynamics has closely been monitored on a monthly basis by the Ifakara Centre Demographic Surveillance System (IC-DSS) since 1996.

Study subjects

The ethical clearance was obtained from both National Institute for Medical Research (NIMR) and IHRDC Institutional Ethics Committee authorities. We obtained informed consent from parents or guardians of participating children. Blood samples were collected between January and August 2002. About 172 patients consisting of males and females with uncomplicated *falciparum* malaria and aged 6 – 69 months were initially recruited in this study using the WHO-set criteria described by the MIM/TDR guidelines for protocols involving molecular markers for drug resistance and multiplicity of infections (2001). SP was given as a single dose on day 0 equivalent to 25 mg/kg body weight based on sulphadoxine component (WHO, 2001). Fractions of the tablets were rounded up to the nearest quarter. Thirty-one patients were either excluded from the study due to failure to comply with criteria for participating in the study or were lost during follow up. Consequently 141 blood samples were available and were used in molecular analysis.

Sample collection

Blood samples for parasite genotyping were collected on filter paper (3MM Whatman), labeled and identified, and kept in a dry clean container with desiccant for a minimum of 3 h to dry. Dry filter paper blood samples were stored at room temperature until when were analysed. The follow-up samples were obtained at days 3, 7 and day 14 after SP treatment. Additional follow-ups were done at any other day if the child was unwell. During all these visits, finger-prick blood was obtained for microscopy and genotyping.

DNA Extraction and PCR analysis

P. falciparum DNA was extracted from blood collected on 3MM Whatmann filter paper by chelex method as previously described (Plowe et al., 1995). The extracted DNA from each sample was used immediately for PCR and the remaining portion was kept frozen at -20ºC. The DNA was amplified in two steps, the primary and nested PCR as described by Foley et al. (1992), Duraisingh et al. (1998) and Felger et al. (1999) . Primers sequences and PCR conditions are shown in Table 1. Amplification was repeated at least twice before the sample was declared negative. The primary and secondary PCR reactions were done in 25 µl and 30 µl final volumes, respectively, and the final volume contained 250 nM primers, 1.5 mM $MgCl_2$, 125 µM of each deoxyribonucleoside tri-

Table 1. Table of PCR primer sequences and reaction conditions for the nested amplification of MSP2 gene.

Primer		Primer sequence	PCR conditions
Outer	S2	5’ GAGGGATGTTGCTGCTCCACAG 3’	94°C-3 min, 94°C-30 s,
	S3	5’ GAAGGTAATTAAAACATTGTC 3’	42°C-60 s, 65C-2 min, x30, 72°C-3 min, 4°C-hold
Inner	S1	5’ GAGTATAAGGAGAAGTATG 3’	94°C-30 s, 50 ^{O}C-60 s,
	S4	5’ CTAGAACCATGCATATGTCC 3’	72°C-2 min, x30, 72°C-3 min, 4°C-hold

Source: Foley et al., 1992; Felger et al., 1993.

phosphate (dNTPs) and 0.02 U/ µl Taq polymerase (Promega). In primary reaction mixture, 5 µl of template DNA was used while in the nested PCR reaction; aliquots of 2 µl of primary PCR products were added. For controls, purified genomic DNA from HB3, 3D7, 7G8 and V1S laboratory strains were used.

Restriction enzyme digestion

The MSP2 nested PCR amplicons were subjected to site-specific restriction enzyme digestion using *Hinf*I enzyme (New England Biolabs). Restriction digestion was performed in 1.5 ml tubes as per manufacturer's recommendations. Essentially 25 l reaction volume was used containing 17 l aliquot of reactants from master mix (1x restriction buffer, restriction enzyme and sterile water [Sigma chemicals Co. ST Luis USA]) and 8 l of PCR product. The reaction mixture was overlaid with mineral oil and capped to prevent evaporation. The restriction digests were incubated overnight at 37^{0}C. To stop the reaction, 5 l of 6x loading buffer (Sigma chemicals Co. ST Luis USA) was added and the reaction mixtures were subsequently preserved at 4^{0}C before gel electrophoresis.

Gel electrophoresis

Gel electrophoresis was performed on nested PCR products to confirm the presence of PCR amplicons of interest. These were electrophoresed on 2% agarose gels in 100 ml of 1x TBE (100mM Tris, 100 mM Borate and 5 mM EDTA) solution. The gels were stained with 0.5 g/ml ethidium bromide final concentration and DNA visualized by UV transillumination. Fragments obtained were compared by size with reference to 1 Kb DNA ladder (Gibco BRL Life Technologies).

On the other hand, polyacrylamide gel electrophoresis was performed on restriction digests as described by Sambrook et al. (1989). This was done on 10% polyacrylamide gel (PAA) using 1x TBE and 1Kb DNA ladder (Gibco BRL Life Technologies). Electrophoresis was carried out at a constant voltage of 11.25 V/cm gel for 2.30 h and the gel stained with ethidium bromide and visualized under UV light.

Statistical analysis

The PCR-RFLP data was analysed using the EPI Info Version 6.04 epidemiological software (Centre for Disease Control and Prevention, Atlanta, GA, USA). The prevalence of each allelic family in the MSP2 gene was calculated as the percentage of baseline (D0) samples containing alleles of that particular family (Aubouy et al., 2003). The distribution of specific families was estimated as the percentage of fragments assigned to one family within the overall number of fragments. The multiplicity of infections and parasite diversity was found by counting and comparing the number of different alleles within and between the two allelic families basing on the different restriction fragment sizes (Felger et al., 1999). In this context, multiplicity of infection (MOI) was defined as the number of genotypes per infection and was calculated as the highest number of genotypes at the MSP2 locus of the parasite DNA and was the basis for establishment of parasite diversity (Aubouy et al., 2003).

RESULTS

PCR amplification of the MSP2 gene

From clinical evaluation (clinical data provided by IHRDC) a total of 172 children with uncomplicated malaria were recruited into the study and treated with SP. Out of these recruited patients, 141 (82%) successfully completed the study. Data from 31 (18%) patients who could not complete the 14 days follow-up were excluded from analysis. Treatment failure was depicted in 15 (10.6%) patients of which, 6.7% was early treatment failure and 3.9% was late treatment failure. Adequate clinical response was observed in 126 (89.4%) of patients. Successful PCR amplification of MSP2 gene was achieved in 125 (88.7%) out of 141 samples analysed while 16 (11.3%) samples were PCR negative. Figure 1 and 2 depicts the PCR amplification of the MSP2 gene using S1/S4 primers of some representative samples. The band sizes of PCR amplicons ranges between 378 and 740 bp.

Detection of multiplicity of *P. falciparum* infection by RFLP analysis

The number of infecting genotypes in an isolate (multiplicity of infection) was detected by restriction digestion (RFLP) of the MSP2 gene (Beck et al., 1997) using *Hinf*I (New England Biolabs) followed by PAA gel (10%) analysis. Restriction digestion was successful in 81% (101/125) of PCR products. Figure 3 depicts representative restriction fragment patterns of the MSP2 gene obtained. Products contained conserved fragments of sizes 115 and 137 bp for FC27-type alleles and variable fragments ranging from 150 – 378 bp. For 3D7-type alleles, conserved fragments of 70 and 108 bp and variable fragments of sizes ranging from 250 to 550 bp

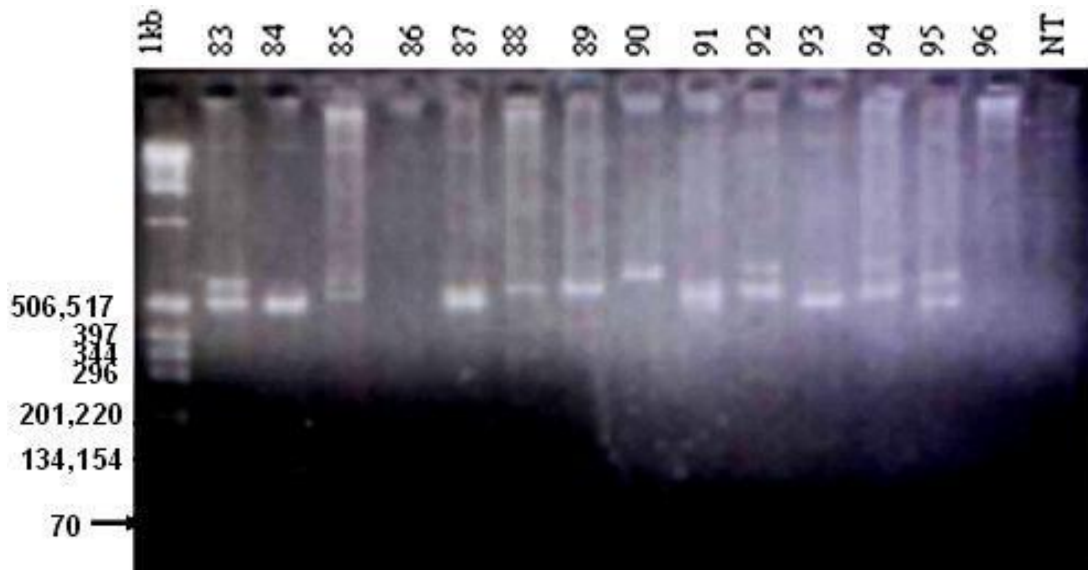

Figure 1. MSP2 gene PCR products run on 2% agarose gel. Number 86 and 96 indicate negative DNA samples. NT = negative control sample. PCR band sizes lies between 378 and 740 bp. An arrow indicates the position of a 70 bp band.

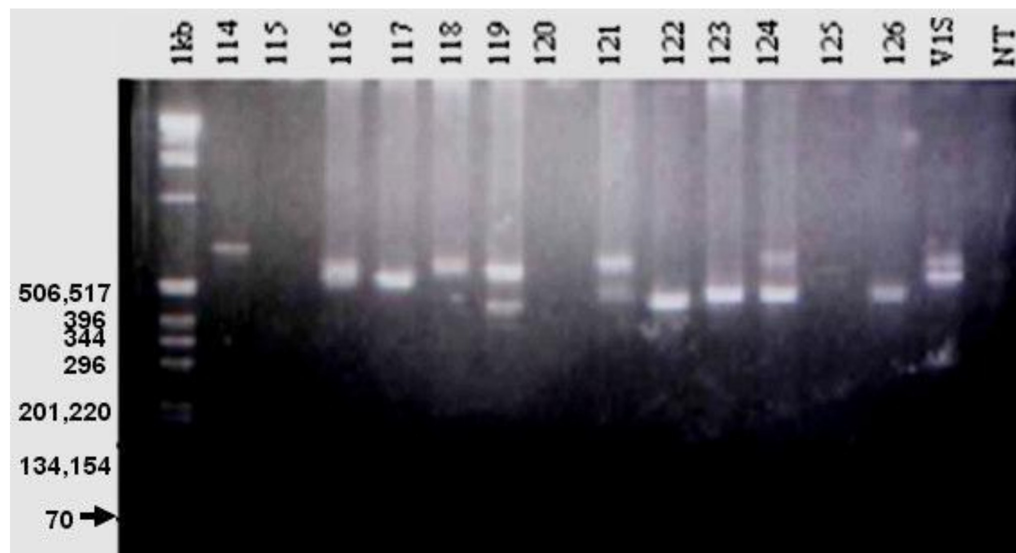

Figure 2. MSP2 gene PCR products run on 2% agarose gel. Number 114 to 126 indicate positive DNA samples. VIS = positive control and NT = negative control. Samples number 115, 120 and 125 were negative. PCR bands lies between 378 – 740 bp.

were obtained (Figure 3 and 4).

Furthermore, considering individual allelic families (Table 2), 75 (74.3%) patients carried 3D7 allelic family genotypes in which 57 (56.4%) occurred as single alleles. Double 3D7 allelic family genotypes were detected in 14 (13.9%) while 3 (3.0%) had triple 3D7 genotypes. Only 1 (1.0%) possessed quintuple genotypes. On the other hand, the FC27 allelic family occurred in 62 (61.4%) infants in which 47 (46.7%) were single genotypes and 12 (11.9%) were double genotypes. Two (2.0%) of the infants had triple FC27 genotypes and 1 (1.0%) had quadruple genotypes (Table 2). In this particular study, multiplicity of infections of above three infections per patient was defined as multiple infections.

Overall multiplicity of infections of the MSP2 genotypes of the *P. falciparum* 3D7 and FC27 allelic families from Mlimba are depicted in Table 3. It was observed that, 51 (50.5%) out of 101 infants carried single infection of either of the two allelic families. Double infection occurred in 32 (31.7%) infants. Twelve (11.9%) infants carried trip-

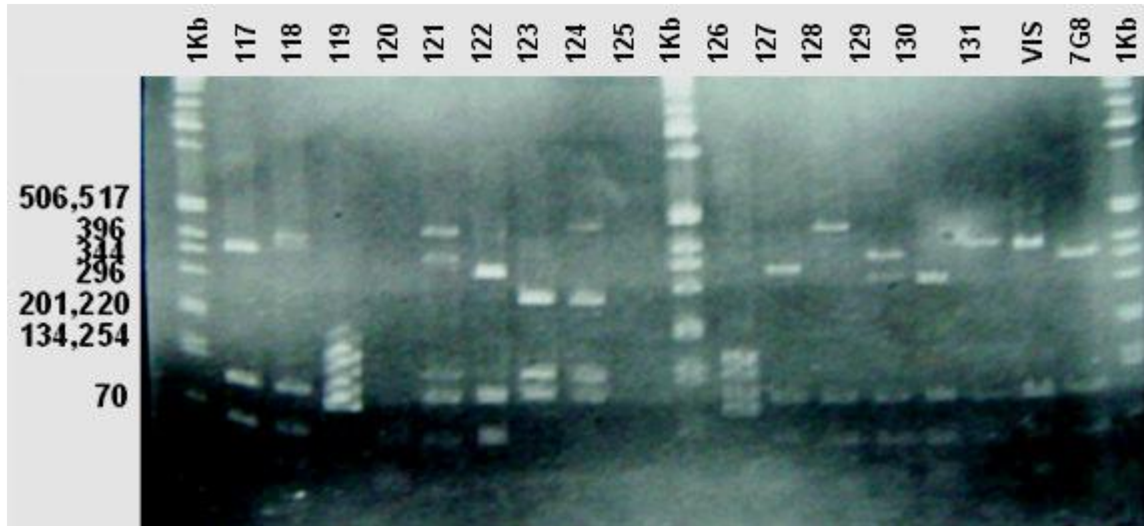

Figure 3. MSP2 RFLP products on 10% PAA gel. Lanes 122 and 123 represent the 3D7 and FC27 allelic families, respectively. The patterns of fragment sizes are 70, 108, 290 bp for 3D7 and 115, 137, 260 for FC27 in that order. Lanes 119 and 126 indicate degraded DNA fragments.

Table 2. Frequency of different MSP2 alleles of *P. falciparum* found in 101 PCR-RFLP successful samples of infants from Mlimba.

Allelic Family	(Allele)'	Single Infection	Double Infection	Triple Infection	Multiple Infection	Total
3D7	26 (25.7%)	57 (56.4%)	14 (13.9%)	3 (3.0%)	1 (1.0%)	101 (100.0%)
FC27	39 (38.6%)	47 (46.5%)	12 (11.9%)	2 (2.0)	1 (1.0%)	101 (100.0%)
Total	65	104	36	5	2	212

(Allele)' = Not of that particular allelic family but the counterpart (Allelic family complement)
Infection is defined by various genotypes of the two allelic families as obtained from PCR-RFLP analysis.

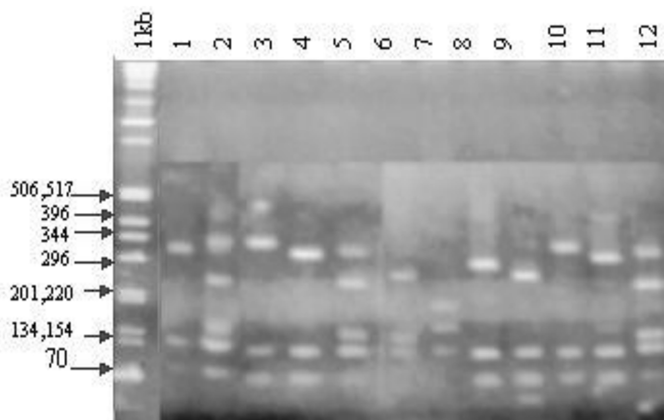

Figure 4. A PAA representative gel for MSP2 PCR-RFLP. Restriction patterns in lanes 2, 5 and 12 represent samples with mixed genotypes in a single patient defining the multiplicity of infections per infection.

le infection and six (5.9%) had multiple infections. Multiplicity of infection ranged from 1 to 6 infections per blood sample with mean multiplicity of 2.58 per infection

Table 3. Overall frequency of multiplicity of infections of the MSP2 genotypes of the *P. falciparum* 3D7 and FC27 allelic families from Mlimba.

Infections	Frequency	Percent	Cumulative Frequency
Single	51	50.5%	50.5%
Double	32	31.7%	82.2%
Triple	12	11.9%	94.1%
Multiple	6	5.9%	100.0%
Total	**101**	**100.0%**	

excluding single infections. When single infections were included in estimating the multiplicity of infection, an average multiplicity of 1.78 infections per patient was obtained. Pure 3D7 allelic family genotypes were detected in 39 (38.6%) of all genotypes detected (n = 101). Twenty-six (25.7%) of samples carried purely FC27 allelic family genotypes. About 36 (35.6%) carried mixed alleles of these two allelic families. In summary, we detected about 20 different alleles of the 3D7-type and 16 different FC27-type alleles in this study. The alleles occu-

rred as single or mixed with another or other alleles within and between families pinpointing marked parasite diversity.

Since this was a longitudinal study, restriction digestion was also done on 15 follow- up samples in an attempt to detect recrudescent or new infections. These samples had parasitaemia either at day 3 (D3), day 7 (D7), day 14 (D14) or in all these days of follow up. No DNA fragments were determined in two samples. Presumably, three samples had restriction fragment patterns on D3, D7 and D14 similar to respective D0 samples despite one of them missing similar restriction fragments on D7. One sample had similar RFLP fragments on D3 and D14 which were different from D0 fragments but none on D7. Another sample had detectable RFLP digests only on D0 but not in the rest of follow up days. There was also a sample that had similar D0 and D3 restriction fragment patterns belonging to 3D7 allelic family but in the subsequent two follow up days, double infections were detected one belonging to either of the two MSP2 allelic families. In this sample the original allelic family persisted with a new one belonging to the FC27 allelic family emerging. Another sample had baseline single infection similar to D3 and D7 belonging to 3D7 allelic family but D14 had a different infection belonging to FC27 allelic family. Two samples had similar restriction fragment patterns belonging to FC27 allelic family but none was detectable on the rest of follow up samples (D7 and D14). Restriction digestion analysis also showed existence of a single infection of 3D7-type in D0 and D3 in two samples but none could be detected on D7 and D14 samples. Another sample had triple infections two of 3D7-type and one of FC27 allelic family detectable at D0. In this sample, one of 3D7 allele was not detected on D3 but two, each belonging to either of the two allelic families. D7 sample possessed all triple infections detected at D0. No infection was detectable on D14. One sample harboured a single 3D7-type allele on D0; none could be detected on D3 and D7 and in D14, a single infection belonging to FC27 was detected.

DISCUSSION

The 141 infants aged less than five years recruited in this study, represent the age group most vulnerable to *falciparum* malaria (WHO, 2003). Clinical data reflected treatment failure in 10.6% of patients. This group of patients who successfully completed the study could have been possessing either recrudescent parasites or new infections by D14. We therefore used the clinical observations as a base for planning molecular studies on the causal-outcome relationship regarding SP treatment in follow up samples in children from Mlimba. The treatment failures observed in this study were probably attributable to parasite resistance to SP. This was previously reported by Kyabayinze et al. (2003).

Parasite recurrence in SP treated individuals has been linked to many factors (Felger et al., 1999). Such factors include overwhelmed immunity, multiple concurrent infections and drug resistance. In this study detection of polymorphisms in both the MSP2 and the drug target genes were done in accordance to Felger et al. (1999) and Duraisingh et al. (1998), respectively. Primers used in this study have been used by Foley et al. (1992) and Felger et al. (1993) and are stipulated in 'The Guidelines for Protocols Involving Molecular Markers of Drug Resistance and Multiplicity of Infections' part IV (MIM/TDR, 2001) that was adopted in this study. The standard protocol can also be obtained from http://www.nlm.nih.gov/adrn/sop_molecular_markers.pdf.

To obtain desirable fragment containing the region responsible for polymorphisms in the MSP2 gene, the primers S2/S3 and S1/S4 were used as primary and nested primers, respectively. These primers have been developed to amplify the 5' and 3' conserved region immediately flanking the central polymorphic part of the MSP2 gene (Felger et al., 1999) and produce DNA fragments varying from 378 bp to 740 bp (Figures 1 and 2). The PCR amplification success was high (88.7%) and the PCR failures (11.3%) are probably attributable to low parasitaemia undetectable by PCR, microscopical errors (e.g. wrong determination of *plasmodium species*) or mislabelling of slides or blood samples, sequestration of parasites at time of sampling (Färnert et al., 1997), or due to point mutations at position where the MSP2-specific primers anneal (Kassberger et al., 2002) thus impairing successful results.

*Hinf*I digestion of PCR amplicons produces restriction fragments, distinguishing the 3D7 from FC27 allelic families of MSP2 gene. Separation of the RFLP digests was done on 10% polyacrylamide gel. The frequency of different MSP2 allelic families of *P. falciparum* in children from Mlimba was found to be high in this study. About 51 (50.5%) out of 101 infants carried single infection of either of the two allelic families (3D7 and FC27). Double infection was observed in 32 (31.7%) of the infants, 12 (11.9%) infants carried triple infection and 6 (5.9%) had multiple infections. The number of concurrent genotype per blood sample (multiplicity of infection) in this study showed up to 6 infections per patient with only two patients harbouring six infections per blood sample. About 50 (49.5%) of patients harboured 2 – 6 infections, leading to an average of 2.58 infections per patient. This was slightly lower than the multiplicity of 3.2 – 3.4 per patient previously reported by Babiker et al. (1999) in samples collected from patients in Michenga, a village along Kilombero valley which is endemic to the disease like Mlimba. However, in contrast to that study, multiple infections were found in nearly 50% of the patients in Mlimba when compared with 85% obtained in Michenga. When single infections were included in estimations of multiplicity of infections, an average multiplicity of 1.78 infections per patient was obtained. This implies that on

average each sampled infant in Mlimba division had at least two infections at the time when blood sample was collected.

In many areas, the parasite population changes with time and season. Babiker et al. (1999) reported differences in multiplicity of infection between areas with seasonal transmission and those with stable transmission (endemic areas), the multiplicity being higher in the latter due to genetic recombination. Felger et al. (1999) observed that all PCR products representing recombination between the 3D7 and FC27 allelic families contained family-specific 36 and 96 bp repeats which were classified as FC27. These fragments were detected in this study, even though at low level (19.8%) and were classified as FC27 alleles. In addition, the large proportion of infants with single infection lowered the overall multiplicity of infection in this study when included in the analysis. Multiplicity of infections is also dependent on the typing method and age group of patients from which the blood samples are obtained. Beck et al. (1997) and Smith et al. (1999) reported a higher load of infection among children aged between 1 – 9 years old with multiple infections of about 5 clones per patient. However in another study conducted in the same area by Felger et al. (1999) revealed an average of 2.1 clones among infants, which is close to what was obtained in this study (average of 1.78 clones per infection).

In regard to individual allelic families, this study showed that 75 (74.3%) sampled infants carried 3D7 allelic family in which 57 (56.4%), 14 (13.9%), 3 (3.0%) and 1 (1.0%) of the parasite occurred as single, double, triple and quintuple infections of the respective allelic family (Table 2). The FC27 allelic family occurred in 62 (61.4%) patients in which 47 (46.7%), 12 (11.9%), 2 (2.0%) and 1 (1.0%) were single, double, triple and quadruple infections, respectively. This shows that, of the two polymorphic MSP2 gene allelic families, the 3D7 was more prevalent in the study population than the FC27. The composition of alleles in each family in this study was however, similar to that reported by Felger et al. (1999) who conducted a similar study in the Kilombero valley along which Mlimba is situated. This shows that the parasite population in this area might have become stable which may be attributable to endemicity of the disease in the area resulting to frequent cross-mating between parasites. This is important in disease intervention studies as it allows clones with novel genotypes to be produced, bringing together genes, which additively generate phenotypes such as multiple drug-resistances (Babiker et al., 1999).

Allelic diversity in the MSP2 gene detected in this study was high with 20 different 3D7 family alleles and 16 different FC27 family alleles observed. This indicates that, at the MSP2 gene, 3D7 and FC27 alleles were nearly equally distributed (ratio = 5/4). More or less similar results were reported by Smith et al. (1999) in Namawala, another area lying along the northern edge of flood plains of the Kilombero River.

Data from follow up samples showed that some alleles either transiently disappeared or additional alleles appeared and eventually disappeared again in some samples during follow up. This observation has been reported by Aubouy et al. (2003) in Sudan. In the present study, similar RFLP fragment patterns were observed from samples collected from D0 to D14 in three patients during follow up even though one of the samples had no RFLP digests on D7. The re- occurrence of similar fragments in these three samples may be coupled with a recrudescing parasite. However infection with a new clone of similar genotype is not uncommon in a stable parasite population due to cross-mating as was reported in previous studies by Babiker et al. (1999). The absence of restriction digests in one sample on D7 might be due to absence of parasite DNA sample possibly accounted with low parasitaemia following medication. In multiple infections, the most abundant allele in the blood sample suppresses amplification of an allele, which is less abundant (Contamin et al., 1995). The variable RFLP digests in the rest of follow up samples could be attributable to the exposure of infants to parasite clones with inconsistent susceptibility to SP in endemic areas. In this context, the patients are probably intermittently infected and re-infected with *P. falciparum* strains possessing different sensitivity to the drug constantly circulating between hosts in the parasite population.

In conclusion, our findings showed that the marker genes selected were suitable for use in this study. The malaria parasites in Mlimba were highly polymorphic and diverse due to high number of different alleles detected in MSP2 allelic families. In addition, genotyping of these genes enabled establishment of multiplicity of infections and level of recrudescence in the study area. However, from this study we could not rule out that clonal disappearance during follow up was the result of treatment activity, and its reappearance due to re-infection by new mosquito bite.

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