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Full Length Research Paper

Genetic diversity of *Plasmodium falciparum* in pregnant women in an IPTp setting in the Offinso District of Ghana

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***Plasmodium falciparum,* the most virulent and deadly of all the malaria species is known to be genetically diverse. This cross–sectional study assessed the genetic diversity of *P. falciparum* parasites in pregnant women in the Offinso District. Blood samples of pregnant women, irrespective of their intermittent preventive treatment of malaria in pregnancy (IPTp) status, were examined for parasitaemia and species identified, from October 2005 to June 2007. Blood blot filter mats were prepared from each participant's blood sample and used for parasite DNA extraction and PCR analysis. DNA extraction was carried out for 126 samples using the Tris-EDTA buffer-based method. Nested PCR reactions were performed for each of the three polymorphic markers *msp1*, *msp2* and *glurp*. PCR amplifications were successful in 22, 43 and 56% of samples for *msp1, msp2* and *glurp*, respectively. The *msp1* (11 alleles) and *msp2* (16 alleles) revealed considerably greater parasite diversity than *glurp* (5 alleles). *Msp2* allelic families were more diverse in comparison with *msp1* and *glurp*. The diversity of parasites threatens the effectiveness of using sulphadoxine – pyrimethamine (SP) in IPTp, hence, the need for continuous monitoring to promptly capture any development of resistance to SP.**

Key words: Malaria, *Plasmodium falciparum*, genetic diversity, pregnant woman, intermittent preventive treatment in pregnancy (IPTp).

INTRODUCTION

The complexity of *Plasmodium falciparum* in adapting to changing environments, drug pressure, and host immune response has been the area of recent biomedical research to ascertain the success of the malaria parasite's adaptation (Tongren et al., 2004; Kumkhaek et al., 2005; Chenet et al., 2008).Knowledge of the nature and extent of genetic diversity within the species becomes increasingly relevant as control measures become more sophisticated and more selectively targeted towards the molecular components of the parasite. Populations of *P. falciparum* are known to be genetically diverse, even at low levels of endemicity (Paul et al., 1999; Bendixen et al., 2001). This shows that there is a remarkably high genetic diversity in endemic transmission settings such as Ghana. A study by Jordan et al. (2001), found that parasites of major epidemics associated with abnormal weather and the extensions of

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malaria transmission to other non–malarious areas are also genetically diverse. The present study was to assess the genetic diversity of *P. falciparum* parasites in pregnant women in a routine intermittent preventive treatment programme (IPTp) setting in the Offinso District of Ghana.

METHODOLOGY

Collection and selection of samples

Offinso District is one of the 27 administrative districts in the Ashanti Region of Ghana. Pregnant women of 16 weeks gestation to term (irrespective of their IPTp status) were studied in six selected health facilities in the District from October 2005 to June 2007. These health facilities were St. Patrick's Hospital (Offinso), District Assembly Maternity Clinic (Offinso), Nkenkaasu Hospital, Abofour Health Centre, Akomadan Health Centre and A.M.E. Zion Health Centre (Afrancho).

Peripheral blood samples of the pregnant women were blotted on Whatman No.1 filter paper (Whatman International Ltd, Maidstone, England), air dried, placed singly in sealed envelopes and labelled. The filter papers were stored with silica gels in a cool dry place. After microscopy, the corresponding blotted filter samples for the PCR were selected based on whether the samples were positive for parasitaemia or not.

DNA Extraction and PCR Amplification

DNA extraction from the dried blood spots on filter paper was carried out using the Tris-EDTA (TE) buffer-based method (Bereczky et al., 2005). Thus, TE buffer (10 mM Tris, pH 8, 0.1 mM EDTA) was prepared and kept at room temperature (25°C). Two to three punches were made from each filter paper and placed in Eppendorf tubes. The punches were soaked in 65 µl of TE buffer and incubated at 50°C for 15 min. They were then pressed gently at the bottom of the tubes several times, using a new pipette tip for each tube and heated at 97°C for 15 min on a heating block or in a water bath. The tubes were centrifuged shortly for 2 to 10 seconds. The punches were removed from the tubes using new pipette tips for each tube and the eluate (DNA extract) kept at 4°C in a refrigerator for use within a few hours or stored at –20°C.

PCR amplifications were performed using a PTC-100^{TM} thermal cycler (MJ Research Inc., Watertown, USA).

The polymorphic repetitive regions of block 2 of merozoite surface protein 1 (*msp1*) (Miller et al., 1993; Magesa et al., 2001), block 3 of *msp2* (Smythe et al., 1991; Magesa et al., 2001) and glutamate rich protein (*glurp*) region (Borre et al., 1991; Magesa et al., 2001) were amplified by nested PCR to assess the diversity (allelic families) of the *P. falciparum* in the women in the district. Samples from individual patients were run in adjacent lanes. If there was no amplification for any allelic family, the PCR was repeated with two times the quantity of template DNA. If no amplification was detected after this second reaction, amplification was classified as unsuccessful.

The primary and nested (second amplification) PCR reactions were carried out in 25 µl reaction volumes using 5 µl of template DNA and 1 µl of primary PCR product respectively in the two reactions. The allelic family-specific primers were used in the nested reaction for block 2 of *msp1* corresponding to MAD 20, K1 and RO33 allelic families, and FC27 and IC for the central region of *msp2*. The sequences of the primers are listed in Table 1.

Each PCR reaction mix contained 1x PCR buffer, 10 mM of each of dNTP, *Taq* DNA polymerase (1.0 U) and 10 µM of each of the forward and the reverse primers. Genomic DNA from 3D7 laboratory strains and SDH_20 (sterilized distilled water) were used as positive and negative control respectively. One to two drops of mineral oil was added to the PCR mixtures to prevent evaporation during the thermal cycling reactions.

The PCR temperature profiles were as follows:

For *msp1* primary reaction, the initial denaturation was at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 50°C for 35 s and extension at 68°C for 2 min and 30 s, then a final extension cycle at 72°C for 3 min. The nested reaction was done at initial denaturation temperature of 94°C for 2 min followed by 35 cycles of denaturation at 95°C for 1 min annealing at 50°C for 15 s and extension at 72°C for 30 s. There was a final cycle of denaturation at 95°C for 1 min, annealing at 50°C for 15 s and extension at 72°C for 3 min.

For *msp2* primary reaction, the initial denaturation was at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 42°C for 1 min and extension at 65°C for 2 min. The final extension cycle was at 72°C for 3 min. The nested reaction was done at initial denaturation temperature of 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 50°C for 1 min and extension at 70°C for 2 min. The final extension cycle was at 72°C for 3 min.

For *glurp* primary reaction, initial denaturation was at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 25 s, annealing at 45°C for 1 min and extension at 68°C for 2 min. The final extension cycle was at 72°C for 3 min and cooled at 15°C. The nested reaction was done at initial denaturation temperature of 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 70°C for 2 min. The final extension cycle was at 72°C for 3 min.

The nested PCR products were analyzed by electrophoresis using 2% agarose gels. The PCR products were visualized under ultraviolet light after being stained with 0.5 µg/ml ethidium bromide (EtBr) and the results photographed. The results were presented by proportions of yield products and graphs.

Ethical consideration

The aims and objectives of the study were discussed with the District Health Administration, the District Assembly and the opinion leaders in the study communities. Permission to undertake the study was obtained from these stakeholders. Ethical clearance was sought and obtained from the Ghana Health Service and School of Medical Sciences (SMS) Ethics Committees. Each study participant after being briefed and offered the opportunity to ask questions about the study, was provided with individual informed written consent form to sign or thumbprint. The written consent forms and participant information forms were kept separately from the data collection tools.

RESULTS

PCR genotyping was done for 126 purposefully selected parasitaemia positive samples. PCR amplifications were successful in 22, 43 and 56% of samples for *msp1, msp2* and *glurp*, respectively and in 84% of samples when all the three genes were combined.

Table 1. Primers for the primary and nested reactions.

primers	sequences
msp1	
Forward	5' - CTA GAA GCT TTA GAA GAT GCA GTA TTG- 3'
Reverse	5' - CTT AAA TAG TAT TCT AAT TCA AGT GGA TCA- 3'
msp2	
Forward	5' - ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA- 3'
Reverse	5' - CTT TGT TAC CAT CGG TAC ATT CTT- 3'
glurp	
Forward	5' - TGA ATT TGA AGA TGT TCA CAC TGA AC- 3'
Reverse	5' - GTG GAA TTG CTT TTT CTT CAA CAC TAA- 3'
Allelic genes of *msp1*	
M1-2M (MAD20 fragment)	
Forward	5'- AAA TGA AGG AAC AAG TGG AAC AGC TGT TAC -3'
Reverse	5'- ATC TGA AGG ATT TGT ACG TCT TGA ATT ACC -3'
M1-2K (K1 fragment)	
Forward	5'- AAA TGA AGA AGA AAT TAC TAC AAA AGG TGC-3'
Reverse	5'- GCT TGC ATC AGC TGG AGG GCT TGC ACC AGA -3'
M1-2R (RO33 fragment)	
Forward	5' - TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG- 3'
Reverse	5' - CAT CTG AAG GAT TTG CAG CAC CTG GAG ATC - 3'
Allelic genes of *msp2*	
M2- FC (FC27 fragment)	
Forward	5' - AAT ACT AAG AGT GTA GGT GCA (AG)AT GCT CCA- 3'
Reverse	5' - TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC- 3'
M2-IC (IC fragment)	
Forward	5' - AGA AGT ATG GCA GAA AGT AA(GT) CCT (CT) CT ACT- 3'
Reverse	5' - GAT TGT AAT TCG GGG GAT TCA GTT TGT TCG- 3'
glurp genes	
Forward	5' - TGA ATT TGA AGA TGT TCA CAC TGA AC- 3'
Reverse	5' - GTG GAA TTG CTT TTT CTT CAA CAC TAA- 3'

To estimate the genetic diversity of *msp1, msp2,* and *glurp* in the parasite population, the frequency distribution of alleles were determined for the studied samples (Figures 1, 2 and 3).

Msp1 (11 alleles) and *msp2* (16 alleles) revealed considerably greater parasite diversity than *glurp* (5 genes) (Figures 1, 2 and 3). In each sample, *msp2* alleles especially the FC alleles exhibited more parasite diversity as compared to the other markers (Figures 2, 4, 5 and 6). The majority of the samples analyzed in *msp2* harboured ≥ 2 parasite lines. Undifferentiated parasite lines of 2 were common in most (98%) of the samples for *glurp* genes hence it showed lesser degree of polymorphism (Figure 6). However, *glurp* genes were highly (56%) detected in the pregnant women as compared with *msp* 2 (43%) and *msp1* (22%) markers.

DISCUSSION

Plasmodium falciparum is genetically very diverse (Paul et al., 1999; Bendixen et al., 2001). In our study in the Offinso District of Ghana, diversity of the parasite was also encountered. *Msp1* and *msp2* were found to be more diverse as compared to *glurp* genes although the later was the most frequently found gene in the parasites infecting the pregnant women studied. FC alleles of the *msp2* in the samples studied were extensively variable as compared with the IC alleles. Those of the *msp1* polymorphic genome were less diverse in comparison. Thus, alleles of the *msp2* genome were highly diverse in the pregnant women as compared to the *msp1* alleles in the pregnant women in the district. This highly diverse nature of *P. falciparum* is indicative of a great potential for

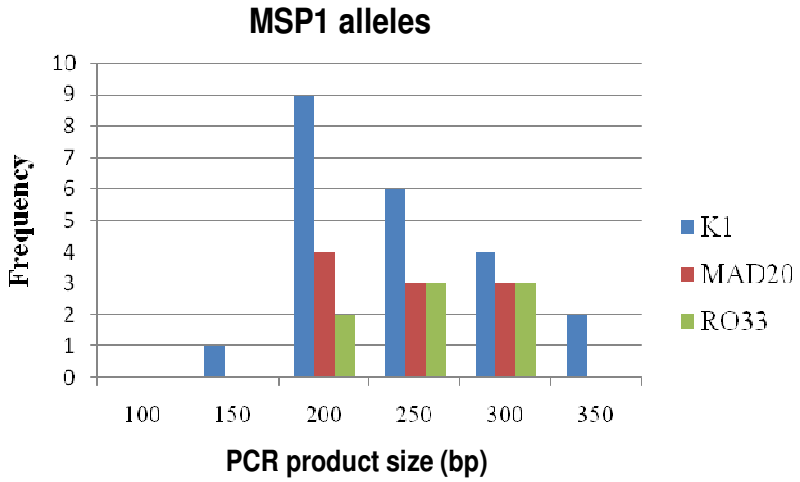

Figure 1. Frequency distribution of MSP1 alleles.

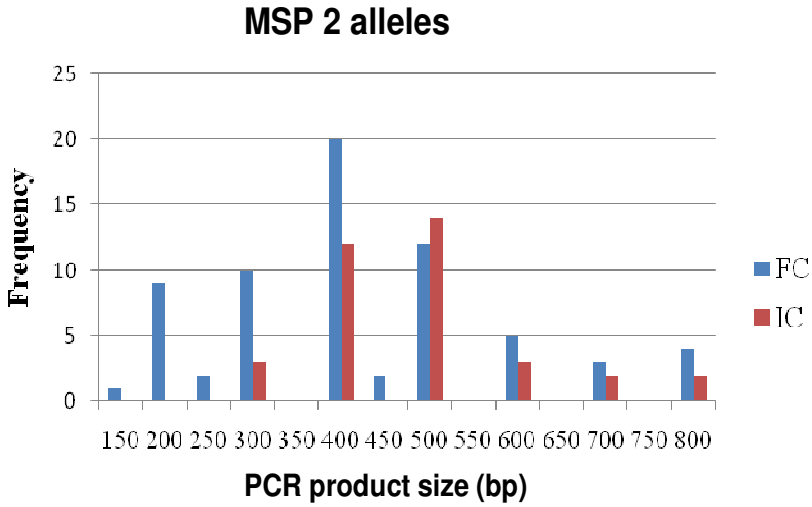

Figure 2. Frequency distribution of MSP2 alleles.

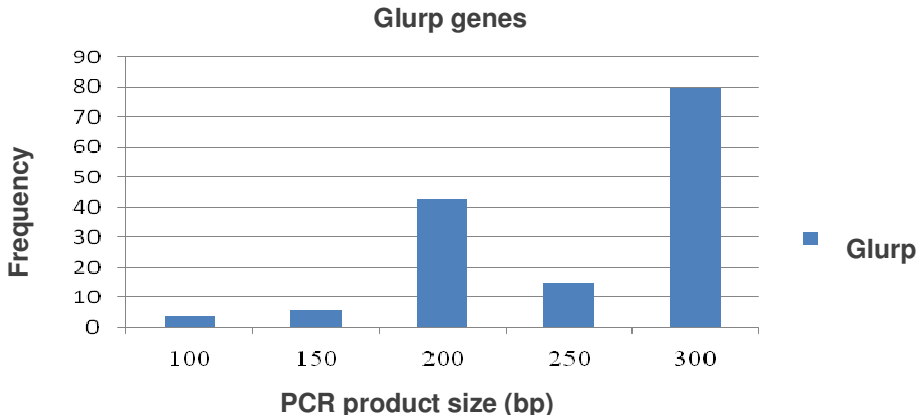

Figure 3. Frequency distribution of glurp genes.

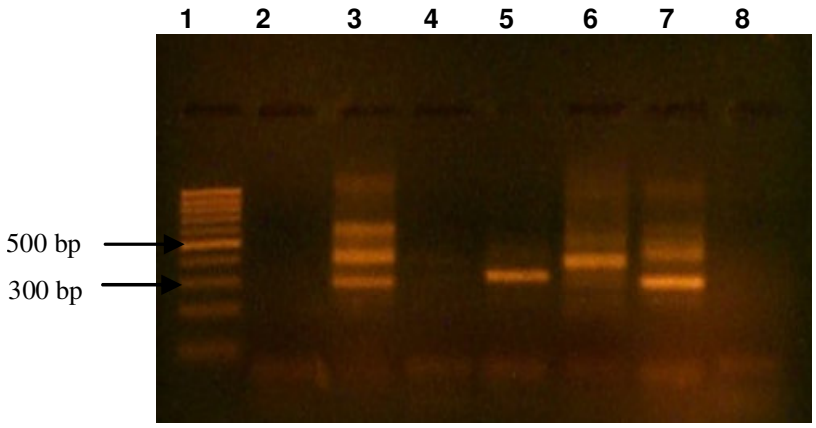

Figure 4. Electrophoregram of MSP2 PCR products of *P. falciparum* malaria: Lane 1 is a 100 base pair DNA marker; DNA fragments in lanes 3, 4(bands faint), 5, 6 and 7 are diverse MSP2 gene FC alleles. Lanes 2 and 8 showed no presence of DNA fragments.

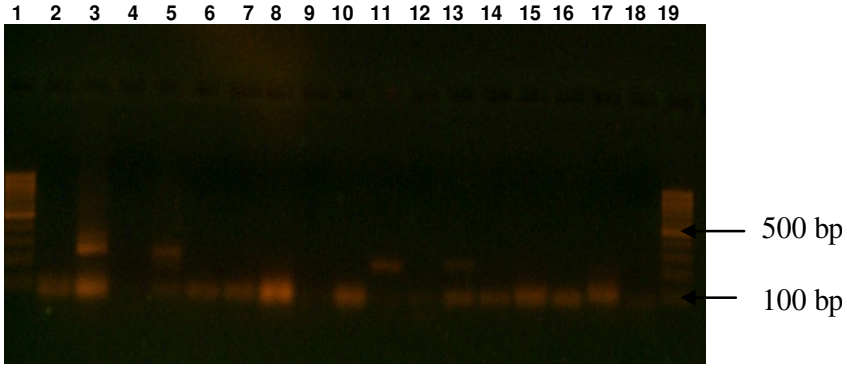

Figure 5. Electrophoregram of MSP1 PCR products of *P. falciparum* malaria: Lanes 1and 19 are 100 base pair DNA markers; DNA fragments in lanes 3, 5, 11, 13 are diverse MSP1 gene K1 alleles.

the development of resistance to SP which may render it less effective as a preventive treatment option. Studies have shown that there is genetic recombination during the sexual phase of the life cycle of the parasite and this enhances the diverse nature of the parasite (Conway et al., 1999; Cano et al., 2007). Hence, malaria control measures must include drug resistance monitoring and assessment of parasite genetic diversity as a means of ensuring success of the control programmes.

The genetic diversity of the parasite in the district suggests high transmission of malaria as a result of high prevalence of multiple infections as reported in other studies (Konate et al., 1999; Paganotti et al., 2004; Cano et al., 2007; Schoepflin et al., 2009). The great diversity of the parasite, thus, poses a potential threat to the effectiveness of SP. In the medium to long term, other antimalarial drugs must be studied as alternatives to SP for IPTp programmes to control malaria in pregnancy in Ghana. There is also the need to introduce efficient vector control measures as part of the overall malaria control strategy in order to reduce the population heterogeneity of the parasite. Malaria control, with a strong vector control component, must also include strong effective health education and behavioural change communication programmes in order to ensure positive outcomes in terms of reduced malaria morbidity and mortality.

Study limitations

Sequencing of the variable regions of the different genes was not done and this might have underestimated the number of alleles and to a variable extent the different

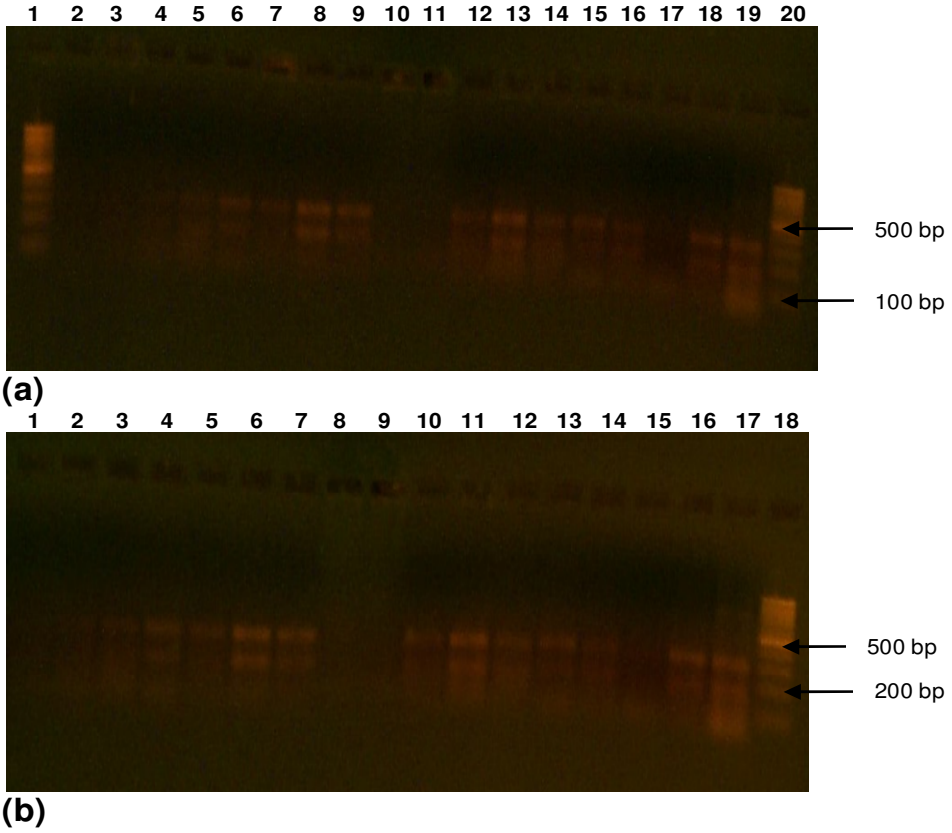

Figure 6. Electrophoregram of glurp PCR products of *P. falciparum* malaria showing less degree of polymorphism with mostly undifferentiated parasite lines of 2: (a) Lanes1 and 20 show double DNA markers of 100 base pair; lanes 8 and 13 show some of the DNA fragments of glurp genes. (b) Lane 18 shows a single DNA marker of 100 base pair; lanes 6 and 11 show some of the DNA fragments of glurp genes.

genes, however, this does not distort the outcome of the present study.

Conclusion

Very diverse genetic strains of *P. falciparum* are infecting the pregnant women in the Offinso District, Ghana. *Msp2* allelic families were more diverse compared to *msp1* and *glurp* in the women. However, from the present study, *glurp* genes were the most common in the women in the district. Antimalarial drug resistance surveillance needs to be a strong component of the current National Malaria Control Programme based on the evidence from this study.

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