Genetic Diversity of *Plasmodium falciparum* Strains in Children under Five Years of Age in Southeastern Tanzania

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Abstract: Strain diversity may play a role in delaying development of protective immunity in endemic areas. We evaluated genetic diversity of *Plasmodium falciparum* infected children before being treated with Sulphadoxine Pyrimethamine (SP) and Coartem™ in Southeastern Tanzania. Allelic diversity of *P. falciparum* strains were determined in order to further assist in correct estimation of recrudescent and new infections.

P. falciparum isolated from 300 children aged 1-59 months was used in the study, where nested PCR followed by Restriction Fragment Length Polymorphism (RFLP) of highly polymorphic Merozoite surface protein 2 (*msp2*) was employed to understand the genetic diversity of the parasites population. Frequency of *msp2* gene alleles was calculated and further associated with multiplicity of infection of children under five years of age.

A total of 71 and 83 different *msp*2 alleles were found in Rufiji and Ulanga districts respectively. Children infected with either FC27 or 3D7 allelic type in Rufiji were 42% single, 55.3% double and 2.7% triple, while in Ulanga, 36.7% single, 62% double and 1.3% triple infections. Mean numbers of multiplicity of infections (MOI) in Rufiji and Ulanga were 1.6 and 1.3, respectively. These findings show a high genetic diversity of *P. falciparum* strains in study areas and low MOI could reflect production of susceptible parasites that immune response can accommodate or can be cleared by the drugs. Furthermore, 3D7 allelic type of *msp2* gene was more prevalent than FC27 in Ulanga district, indicating association between *msp2* allelic type and disease severity, hence predict possible vaccine candidate in the future.

Keywords: *P. falciparum*, genetic diversity, *msp2* gene, Tanzania.

INTRODUCTION

High malaria transmission in many parts of the world has been associated with high *Plasmodium falciparum* genetic diversity [1, 2]. Genetic diversity as a result of genetic recombination increases the risk of an individual to be infected with different parasite genotypes even if the area has stable malaria transmission. Such infections are generally described as complex or multiple infections [2] and have previously been explored on grounds of genetic variation and its effects on parasite population dynamics [3].

Understanding the patterns and mechanisms of DNA sequence variation in major *P. falciparum* surface antigens is critical for the purpose of predicting effectiveness of human host immune response [4]. Moreover, various studies have shown that most endemic areas inhabited by *P. falciparum* are infected with mixtures of genetically distinct clones [5]. It has also been reported that high polymorphism featured in certain surface proteins such as Merozoite surface proteins (MSP) of *P. falciparum* has even hindered vaccine development [6].

Merozoite surface protein 1 (MSP1), Merozoite surface protein 2 (MSP2) and glutamate-rich protein (GLURP) are the most commonly used markers for identifying *P. falciparum* populations diversity. The *msp2* gene has been extensively studied and is so far the most informative single diversity marker [7]. Its polymorphic features have been exploited for studying naturally occurring parasite populations [7, 8]. The *msp2* alleles generally fall into two allelic families, FC27 and 3D7, which differ considerably by the dimorphic structure of the variable central region [9]. Studies conducted in Central African Republic and other areas suggested that, the epidemiological features of *P. falciparum* surface proteins may vary significantly within areas [10, 11]. Furthermore, several studies have associated parasite diversity with treatment outcome [12] but this might not be the case in endemic areas with stable transmission where the immune system can play a major role in parasite clearance or reduction of severity [13, 14].

Here we report the genetic diversity of *P. falciparum* strains among children and apparent number of concurrent infections per blood sample of an individual (MOI) before treatment with Sulfadoxine-Pyrimethamine (SP) and Artemisinin-based Combination Therapy. We enrolled children aged less than five years from Ulanga and Rufiji districts, where as molecular techniques were used so as to provide evidence on genetic diversity status of the parasite strains in these areas and particularly the study population.

MATERIALS AND METHODS

The study was carried out in Southeastern Tanzania in Kibiti, Rufiji district and Lupiro in Ulanga district. It was a cross-sectional study conducted in 2004 as part of the big

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project that aimed to assess combination therapy among children with uncomplicated malaria. Rufiji is a rural district located 178 km south of Dar es Salaam along the Indian Ocean coast with an area of 14,500 km^2 and a population of 203,102. Just like the former, Ulanga is also a rural district but this is found, along the Kilombero river valley about 500 km Southeast of Dar es Salaam with a population of 194,209 [15]. Malaria transmission in Rufiji and Ulanga districts is intense and perennial. The catchment populations at the two health centers in Kibiti-Rufiji and Lupiro-Ulanga were 26,822 and 40,385 respectively.

Ethical Issues

The study obtained ethical clearance from both Institution Review Board (IRB) and national Institute for Medical research (NIMR) ethical committee authorities with the project license No.1810. Oral and written informed consent about risks and benefits that may occur were presented to the parents or guardians of the participating children. The parents and guardians accepted and gave informed consent for the participation in the study.

Sample Collection

We enrolled children aged less than five years from Ulanga and Rufiji districts and randomly selected a total of 300 children with uncomplicated malaria before treatment from the two districts. A total of 150 samples were collected from each district, Ulanga and Rufiji. Thereafter, blood samples were spotted on filter papers (3MM Whatman) in a sterile manner, dried and properly stored at room temperature until DNA isolation and molecular analysis was done. Polymerase Chain Reaction (PCR) as a molecular technique was performed followed by restriction fragment length analysis (PCR-RFLP) of *msp2* genes on DNA isolates. Blood samples were collected before drug administration according to WHO *in vivo* standard protocol [16].

DNA Extraction and PCR-RFLP

P. falciparum genomic DNA was extracted from blood collected on filter paper by modified Chelex method as previously described [17]. The extracted DNA from each sample was used immediately for PCR and the remaining portion was stored at -20°C in appropriately labeled storage tubes. Nested PCR followed by RFLP analysis was carried out to amplify *msp2* gene using primer sets and restriction enzyme for allelic families analysis as previously detailed by Felger *et al.* 1999 [18]. Restricted PCR fragments were sized using DNA marker (Solis BioDyne) as a reference. The reference DNA marker is also known as DNA ladder as its fragment sizes increase from the bottom and hence makes sizing of the PCR fragments easy.

Statistical Analysis

Data were analyzed using Chi-square test and Shannon-weaver index of diversity. Chi-square test was used to compare the allelic families of *msp2* gene in the two districts [18]. MOI was performed using Shannon-weaver index of diversity as detailed in Hutcheson, 1970 [19] but with some modifications.

RESULTS

All 300 blood samples from the study population were successfully analyzed for parasite genetic diversity and MOI. In Rufiji, 40% (60/150) were FC27, 43% (65/150) 3D7 and 17% (25/150) mixed infections, whereas in Ulanga 36.7% (55/150) were FC27, 56% (84/150) 3D7 and 7.3% (11/150) mixed infections. There was a significant difference in distribution of 3D7 and FC27 in Ulanga district (P <0.05) but not in Rufiji (P >0.05).

MOI in children from Rufiji and Ulanga were as follows; Children with single infections of either FC27 or 3D7 allelic type in Rufiji were 42% (63/150), those with double infections were 55.3% (83/150), and 2.7% (4/150) had triple infections. In Ulanga, 36.7% (55/150) single, 62% (93/150) double and 1.3% (2/150) triple infections (Fig. **1**). The mean numbers of MOI were 1.6 and 1.3 in Rufiji and Ulanga, respectively, with a range of 1-3 genotypes per individual.

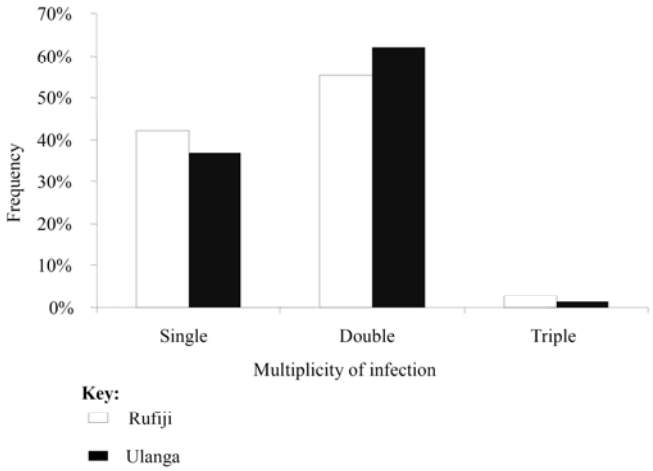

Fig. (1). Frequency distribution of concurrent infections among children under five years of age in Rufiji and Ulanga districts.

Out of 150 infected children from Rufiji, 71 different *msp2* alleles were detected, 36 alleles belonged to 3D7 and 35 belonged to FC27 with no statistical difference between the two families (df = 70, P > 0.05). On the other hand, out of the 83 different *msp2* alleles detected in Ulanga, 45 alleles belonged to 3D7 and 38 belonged to FC27 with clear significant difference between the two families (df = 83, P > 0.05). A total of 69 children both in Ulanga and Rufiji districts were infected with parasites containing 3D7 and FC27 alleles having base pairs of 210, 270, 300, 350 and 400 (Tables **1** and **2**). This indicates that, amplicons of the same size can be found in the two allelic families (3D7 and FC27). This may lead to difficulties in distinguishing recrudescence and new infection basing on these amplicons.

DISCUSSION

In this study we found 71 and 83 different *msp*2 alleles in study samples collected from Rufiji and Ulanga respectively, with 3D7 allelic types featuring the most followed by FC27 in both districts. Genetic diversity of *P. falciparum* strains in the study population was found to be high in both districts which indicate that close ecologically related areas would have similar distribution of parasite strains. This may have an implication on treatment of malaria among children living

Table 1. The Allelic Diversity of *P. falciparum msp2* Allelic Families Occurred in Rufiji

Alleles of 3D7(bp)	Frequency (fi)	fi log fi	fi log2 fi	Alleles of FC27(bp)	Frequency (fi)	fi log fi	fi log2 fi
3D7200	3	1.4314	0.6829	FC27210	6	3.4949	2.4428
3D7370	3	1.4314	0.6829	FC27170	4	2.4082	1.4499
3D7400	4	2.4082	1.4499	FC27300	3	1.4314	0.6829
3D7300	5	3.4949	2.4428	FC27430	2	0.6021	0.1812
3D7500	1	0	0	FC27340	3	1.4314	0.6829
3D7210	4	2.4082	1.4499	FC27270	4	2.4082	1.4499
3D7360	4	2.4082	1.4499	FC27450	3	1.4314	0.6829
3D7270	3	1.4314	0.6829	FC27120	3	1.4314	0.6829
3D7450	4	2.4082	1.4499	FC27220	4	3.4949	2.4428
3D7350	5	3.4949	2.4428	FC27150	3	1.4314	0.6829
Total	n=36	20.9178	12.7339		n=35	19.5653	11.3811

Table 2. The Allelic Diversity of *P. falciparum msp2* Allelic Families Occurred in Ulanga

Alleles of 3D7(bp)	Freq. (fi)	fi log fi	fi log2 fi	Alleles of FC27(bp)	Freq.(fi)	fi log fi	fi log2 fi
3D7270	5	3.4949	2.4428	FC27120	3	1.4314	0.6829
3D7340	3	1.4314	0.6829	FC27150	4	2.4082	1.4499
3D7370	4	2.4082	1.4499	FC27250	3	1.4314	0.6829
3D7320	2	0.6021	0.1812	FC27210	5	3.4949	2.4428
3D7510	3	0.6021	0.1812	FC27170	3	1.4314	0.6829
3D7210	2	0.6021	0.1812	FC27260	2	0.6021	0.1812
3D7360	3	1.4314	0.6829	FC27245	2	0.6021	0.1812
3D7300	4	2.4082	1.4499	FC27510	1	0	0
3D7400	6	4.6689	3.6331	FC27270	5	3.4949	2.4428
3D7355	4	3.4949	2.4428	FC27310	4	2.4082	1.4499
3D7310	3	1.4314	0.6829	FC27330	3	1.4314	0.6829
3D7350	6	4.6689	3.6331	FC27300	3	1.4314	0.6829
Total	N=45	27.2445	17.6439		n=38	20.1674	11.5623

in endemic areas where immunity is stable and drug resistance issues are to be considered. These findings corroborate with those from Dakar, Senegal [20] and Mlimba, Tanzania [21], which reported 3D7 alleles to be more prevalent than FC27 in those infected with single, double and triple infections. Nevertheless, different proportions of the two allelic families in Ulanga observed in this study corroborate with the findings obtained in Dielmo, Senegal [22, 23] and Sao Tome [24]. This difference of the two allelic families has been proposed to arise due to the presence of considerable heterogeneity in parasite populations [23]. On the other hand, results obtained from Rufiji district in this study indicate that, there is no significant difference in the prevalence of the two families. This is similar to findings reported by previous studies done in Kilombero, Tanzania [18], Ndiop, Senegal [25] and Papua New Guinea [26].

The diversity of *msp2* alleles in Rufiji observed between the families in this study supports suggestion by Felger *et al.* 1999 [18] that, although the genetic diversity is extensive, it is limited by structural constraints or immune selection. In this study we found 63.3% and 58% of the individuals harbor more than one infection in Ulanga and Rufiji respectively, as compared to only 25% obtained in Papua New Guinea [23]. The high prevalence of multiple *P. falciparum* infections being reported by this study is broadly consistent with previous findings from other parts of Tanzania including Michenga village [27].

Furthermore, results from this study show that establishment of the specific DNA fragments is a useful indicator for evaluating genetic diversity of parasite populations. It is important to note that, there are similar PCR amplicon sizes in the two families of which can lead to wrong results if their diversity has not been clearly defined. It will then be a

challenge to distinguish recrudescence from re-infection in settings where amplicons are not sequenced to remove possible discrepancies.

Furthermore in endemic areas, the number of different clones of malaria parasites co-infecting a single host (MOI) can be a useful indicator of the level of transmission and/or the immune status of the host [28]. Increase in transmission levels (Entomological Inoculation Rates) is generally associated with progressive increases in the average number of parasite clones per host [27-29].

Mean MOIs obtained in this study were lower (1.6 and 1.3 in Rufiji and Ulanga, respectively) than those obtained in other areas in Tanzania such as, Kilombero, Mkuranga, Mlimba and Kyela [18, 30]. Felger *et al.* 1999 [18] reported the average MOI of 4 in symptomatic children in Kilombero, while Mugittu *et al.* 2005 [30] recorded 2.5 in asymptomatic children in Mkuranga, Mlimba and Kyela. Studies done by Beck *et al.* 1997 [31] reported a MOI of 5 in Senegal. The reason for this observation could be due to technical problems such as lower detection limits of some clones by PCR at low densities, it can as well be speculated due to difference in bed net usage, difference in transmission and treatment seeking behavior of individuals living in the two areas. In previous studies elsewhere in the world, it has also been demonstrated that multiplicity of infection is age dependent but may differ in areas with malaria transmission [22, 32, 33]. However, this factor was not evaluated in this study since samples and data available were only from children less than five years of age. Generally, our findings stress on the presence of highly diverse *P. falciparum* infections in these areas before treatment and created useful information on the presence of the same DNA fragments in both allelic families of the *msp2* gene.

CONCLUSION

In this study we found a high number of *P. falciparum* strains in symptomatic children in Ulanga and Rufiji districts. Most of these strains harbor double infections although the frequency distribution of the two allelic families varied slightly between the two districts. The high parasite diversity indeed indicates high transmission levels in these areas. We anticipate our findings to provide a baseline to detect and describe the parasitological outcome of vaccine development trials and perhaps elucidate biological effects of the vaccine candidate for malaria. Moreover, our findings will help future studies explore allelic diversity of the parasite strains to correctly distinguish recrudescence from new infection in areas where malaria transmission is intense throughout the year.

AUTHORS' CONTRIBUTION

DS drafted the manuscript, designed the experiment, coordinated data collection and analyzed data. KMH and JPM participated to design the study and manuscript development. SA supervised the work from inception, acquisition of funding, and development of the study protocol on the earlier version. All authors read and approved the final version of the manuscript.

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