Scientia Africana, Vol. 8 (No.2), December, 2009 pp 9-16
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GENETIC VARIANTS OF *PLASMODIUM FALCIPARUM* IN INFECTIVE *ANOPHELES GAMBIAE* S.L. AT A RURAL COMMUNITY IN SOUTHWEST NIGERIA

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Received: May, *2009*
Accepted: *August, 2009*

ABSTRACT
During studies on the epidemiology of malaria at a rural community, Igbo-Ora, Southwest Nigeria, genotyping of Plasmodium falciparum extracted from infective Anopheles gambiae s.l. was undertaken. Circumsporozoite (CSP) ELISA was used on crushes from head-thorax for DNA extraction and PCR amplification for the determination of P. falciparum genotypes on merozoite surface protein-1 and 2 (MSP-1 & 2). Of the 65 infective anophelines, P. falciparum genotypes were positively identified in 41. Mono-infections constituted 73.4% of all infections; the dominant mono-infections on MSP-1 and MSP-2 were MAD20 (18) and IC1 (09) respectively; the rare RO33 (01) was recorded. Double infections were 20.20% (09) with both markers, while only one triple infection was observed on MSP-1. An anopheles was found with two double infections, one on each of the two blocks. Eight of the 12 multiple infections were on MSP-1, five on both MSP-1 and MSP-2. In addition to the multiplicity of proteins in these vectors, size polymorphism was observed in alleles, indicating vector/parasite interactions and environmental variations. These results were compared to those from human sera.

Key words: *Plasmodium falciparum*, multiple infections, allelic families, size polymorphism, *Anopheles gambiae* s.l.

INTRODUCTION
The spread of drug-resistant *Plasmodium falciparum* has hampered therapeutic efforts, a major concern in Africa (White, 1999; WHO, 2005). There is an urgent need for the development of new effective measures, including vaccines (Moree, 2003). Studies have demonstrated that the numbers of malaria parasite strains in an individual are correlated to transmission levels (**Arnot, 1998**; Barbiker *et al.*, 1999). Although multiplicity of infections (MOI) may be an indicator of the immune status (Smith *et al.*, 1999), other studies have shown an inverse relationship (Muller *et al.*, 2001). The targeted polymorphic genes used for genotyping in *P. falciparum* are those coding for merozoite surface protein-1 (MSP-1), merozoite surface protein-2 (MSP-2) and glutamate-rich protein (GLURP) (Ntoumi *et*

al., 1995; Viriyakosol *et al.*, 1995; Färnert *et al.*, 2001; Cattamanchi *et al.*, 2003; Joshi, 2003). Studies utilizing these genes, based on blood samples, have been undertaken in several African countries: Nigeria (Engelbrecht *et al.*, 2000; Amodu *et al.*, 2005; Omosun *et al.*, 2005), Senegal (Robert *et al.*, 1996) and Tanzania (Smith *et al.*, 1999). Simultaneous immuno-epidemiological and entomological studies on malaria were conducted at the hyperendemic community of Igbo-Ora, Southwest Nigeria. This paper describes studies on the genotyping of *P. falciparum* extracted from infective *An. gambiae* s.l., collected during studies on the identification, molecular and genetic characterization of *An. gambiae* s.l. These studies were undertaken to determine whether results from infective mosquitoes on multiplicity of proteins and size polymorphism of alleles in *P. falciparum* correlate with those from human blood.

MATERIAL AND METHODS

Description of the Study Area

Igbo-Ora (7.4333° N; 3.2833° E) is a rural town with an average annual temperature of 26.14°C and an annual rainfall of 1317mm (Encarta, 2005), located 70Km west of Ibadan in the forest- savannah woodland ecocline, southwest Nigeria (Fig 1). It covers an area of approximately 100km^2, with a population of about 60,000 inhabitants, mainly of the Yoruba ethnic group, dispersed in 170 hamlets grouped into the seven villages (Ajegunle, Igbole, Idofin, Igbo-ora, Iberekedo, Packo and Sagaun) (Akpan, 1997). Contrary to the description by Lawrence (1965), the area is strewn with streams that serve as larval breeding pools for *An. gambiae* s.l. A detailed description of the study area has been published (Noutcha and Anumudu, 2009). Malaria is endemic at Igbo-Ora, with a 6-month transmission season (May- October) reaching its peak in August. Malaria prevalence rates of 42% in adults and 33-70% in children, <1-15 years, were recorded during the study period (Nwuba *et al.*, 2002; Omosun *et al.*, 2005).

Sandwich ELISA for *Plasmodium falciparum* Circumsporozoite Antigen Detection

All head-thoraxes of mosquitoes identified morphologically as *An. gambiae* s.l. were analyzed for the detection of the presence of circumsporozoite antigen, to determine the infection rates. The sporozoite antigen was obtained by crushing each mosquito head-thorax in 50µl of blocking buffer NP_{40} and 200µl of Blocking Buffer [0.5% Casein + 0.1N NaOH + PBS (pH=7) + Thimersol ($C_9H_9HgO_2SNa$) +Phenol Red]. The ELISA technique was performed according to methods adapted from Burkott *et al.* (1987) Wirtz *et al.* (1987) and Beier *et al.* (1987).

DNA Extraction for PCR Analyses and Sequencing

Head-thorax crushes from CSP- ELISA were used as *An. gambiae* s.l. DNA extraction source. The DNA of all CSP- positive mosquitoes was heated at 65°C for 10min in a thermolyne dry bath (Barnstead Thermolyne, Dubuque, USA), to liberate parasite DNA for the determination of *P. falciparum* genotypes on merozoite surface proteins-1 and 2 (MSP-1 & MSP-2). The amplified MSP-1 and MSP-2 served as template DNA for allelic families of MSP-1 (K1, MAD20 & RO33) and MSP-2 (IC1 & FC27) respectively. The primers for mosquito DNA amplification and CSP-ELISA assays were purchased from Invitrogen; those for parasite genotyping were purchased from Integrated DNA Technologies (IDT). The method of Scott *et al.* (1993) was used for *An. gambiae* s.l. DNA amplification while for *P. falciparum* DNA genotyping, Tamura and Aotsoka, (1988), modified by Koita (1999) was used.

Scientia Africana, Vol. 8 (No.2), December, 2009 pp 9-16
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Amplified DNAs were migrated on a 2% gel; the DNA bands were observed and photographed on an UVP trans-illuminator. Visible bands were compared to DNA ladder marker VI (0.5-2.1Kb), (Roche Diagnostics, Mannhein, Germany; Indianapolis, USA).

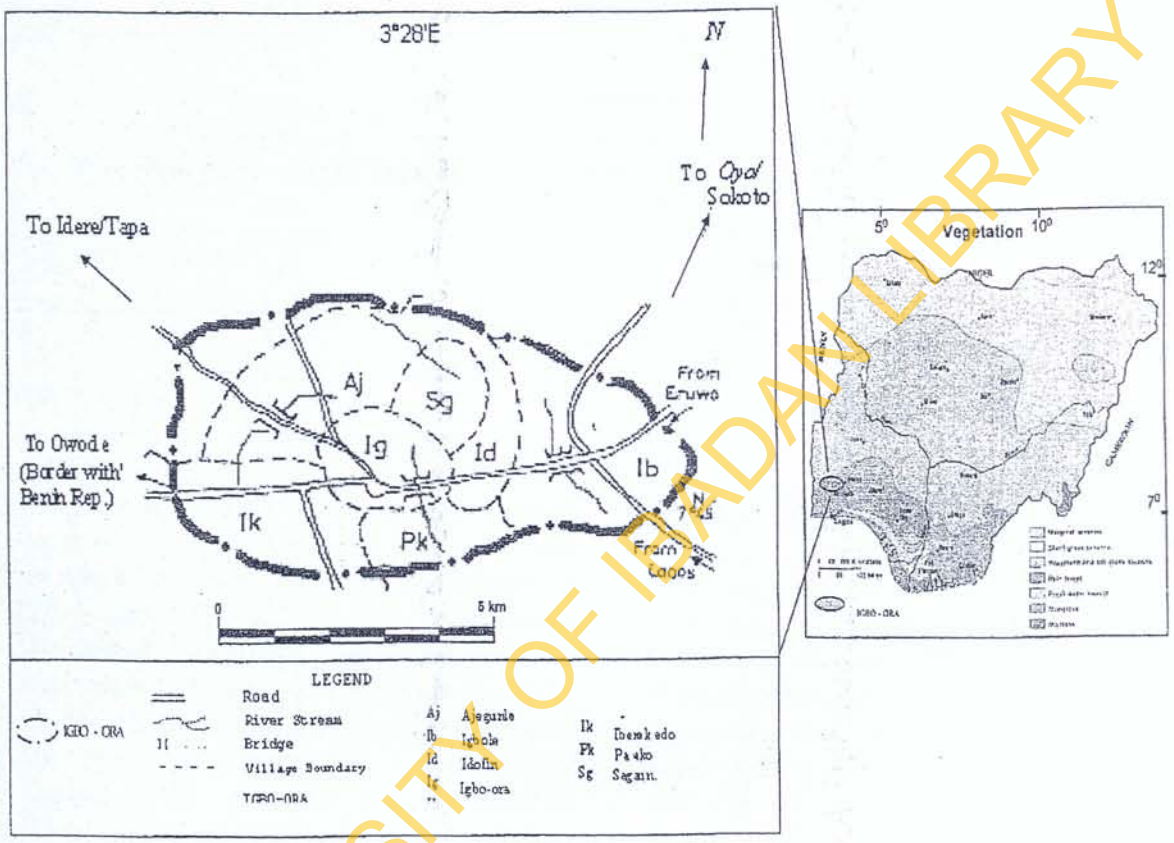

Figure 1: The Collection Sites at Igbo-Ora

RESULTS

Of the 65 infective mosquitoes, *P. falciparum* genotypes were positively identified in 41. Mono-infections constituted 73.4% of all infections, the dominant mono-infection on both blocks was MAD20 (18), followed by IC1 (09), K1 (01) and RO33 (01). Double infections constituted 20.20% (09) of all infections, while only one triple infection was observed; size polymorphism was recorded on three IC1, one FC27 and one Mad20 (Table 1). Eight of the 12 multiple infections were on MSP-1, five on both MSP-1 and MSP-2 and one exclusively on MSP-2 (Table 2).

TABLE 1: SIZE POLYMORPHISM AND MONO/ POLY-INFECTIONS OF *PLASMODIUM FALCIPARUM* MSP1 AND MSP2 AT IGBO-ORA

Mosquito ID	Allelic family	Size (base pair)
0434/21.30	IC1	**369/168**
SQ512.3	IC1	370
0434/21.9	IC1	265
0433/88.2	IC1	323
0433/96.2	IC1	158
0313/36.1	IC1	212
0434/21.9	IC1	232
0434/21.4	IC1	**332/224**
0433/88.2	IC1	**346/270/230**
SQ512.3	IC1/FC27	**369/232/228IC1 419/334FC27**
0313/03.3	FC27	322
0434/18.7	FC27	**503/351**
0313/03A.7	Mad20	230
0313/17.1	Mad20	234
SQ505A.2	Mad20	248
0435/25.7	Mad20	229
0313/36A.17	Mad20	233
0433/96.6	Mad20	254
0313/36A.20	Mad20	257
0434/21.8	Mad20	259
0313/11.2	Mad20	231
0313/36.7	Mad20	225
0313/03.3	Mad20	221
0433/88.8	Mad20	217
SQ512.8	Mad20	213
SQ510.5	Mad20	217
0313/17.10	Mad20	205
0433/36A.9	Mad20	223
SQ003.3	Mad20	**237/199**
0434/21.4	Mad20	227
0313/06.5	K1/Mad20	217/246
SQ501.2	K1/Mad20	213/236
SQ512.3	K1/Mad20	225/232
0434/21.30	K1/Mad20	229/232
0434/18.7	K1	194
0434/24.1	Mad20/RO33	224/158
SQ503A.8	Mad20/RO33	240/174
0435/06.2	Mad20/RO33	232/219
0435/06.3	Mad20/RO33	155/223
0434/21.6	**K1/Mad20/RO33**	**187/241/175**
0434/20.2	RO33	230

Scientia Africana, Vol. 8 (No.2), December, 2009 pp 9-16
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TABLE 2: ANOPHELINES WITH MULTIPLE INFECTIONS AT LOCI 1 AND 2 OF *PLASMODIUM FALCIPARUM* MSP AT IGBO-ORA

Mosquito ID	MSP1	MSP2
0313/03.3	Mad20	FC27
0313/06.5	K1/Mad20	
0434/18.7	K1	FC27
0434/20.2	RO33	
0434/21.30	K1/Mad20	IC1
0434/21.4	Mad20	IC1
0434/24.1	Mad20/RO33	
0435/06.2	Mad20/RO33	
0435/06.3	Mad20/RO33	
SQ501.2	K1/Mad20	
SQ503A.8	Mad20/RO33	
SQ512.3	**K1/Mad20**	**IC1/FC27**
0434/21.6	**K1/Mad20/RO33**	

DISCUSSION

The dominance of mono-infections of *P. falciparum* in mosquitoes was also observed by Arez *et al.*, (2003), who recorded a higher proportion of single genotype infections and less allele diversity. Size polymorphism in MSP-1 and MSP-2 has been extensively documented (Färnert *et al.*, 1999; Cattamanchi *et al.*, 2003; Joshi, 2003). Results from human blood sera, collected from Igbo-Ora during the period of the mosquito study, revealed that on MSP-1, RO33 was the dominant allele, followed by K1 and Mad20 (Ngoundou-Landji, Personal Communication). These alleles were also recorded in the genotyping of *P. falciparum* from infective mosquitoes caught at Igbo-Ora, although not in the same frequency. More multiple infections were recorded in MSP-1 than MSP-2; this is at variance with the studies that found MSP-2 as the most informative single marker for the analyses of MOI (Felger *et al.*, 1999; Färnert *et al.*, 2001). However, the results from human blood at Igbo-Ora, showed more MOIs mainly from MSP-1 (Ngoundou-Landji, Personal Communication). These differences in *P. falciparum* typed from the vector's blood meal and human sera might have resulted from proteomics interactions between the parasite and *An. gambiae* s.l. or humans. Furthermore, the immuno-parasitological and entomological collections were not synchronized. Amodu *et al.* (2005), working at Ibadan, 70Km east of Igbo-Ora, utilized MSP-1 to study the role of genetic characterization of *P. falciparum* in the severity of malaria infections. They showed that the distribution of MSP-1 alleles was significantly different among the following groups: asymptomatic malaria (ASM), uncomplicated malaria (UM) and severe malaria (SM). The absence of k1 alleles was associated with a 3-fold increased risk of UM and a 4-fold increased risk of SM, when compared to ASM. The absence of MAD20 alleles was associated with a 5-fold increased risk of UM and an 8-fold increase of SM. The presence of K1 and MAD20 alleles was significantly associated with ASM and reduced risk in the development of the symptomatic disease. Branch *et al.* (2001) determined that parasitemia with the RO33-genotype was more resistant to subsequent

infections than those without the genotype. The MSPs, involved in erythrocytic invasion, affect parasite density and eventually pathology. One of the main obstacles to the acquisition of anti-malaria immunity is the high degree of antigens, which enable parasites to evade immune responses elicited by past exposure to variant forms of the same antigen (Ferreira *et al.*, 2004).

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